PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: HEPATOCYTE GROWTH FACTOR VARIANTS
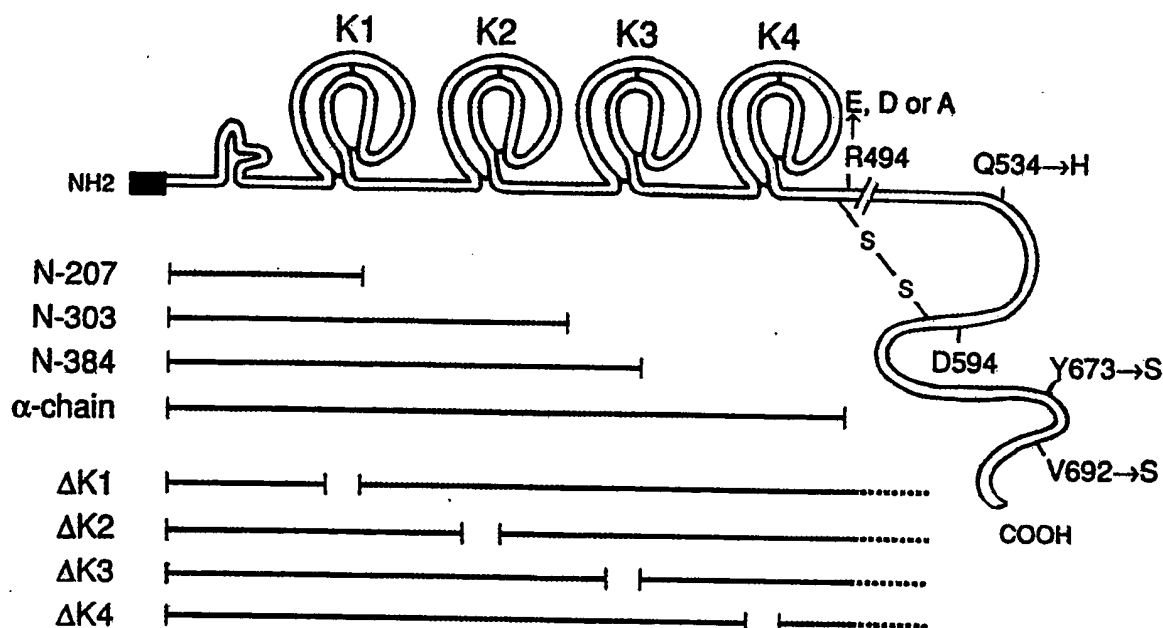
(57) Abstract

The invention concerns hepatocyte growth factor (HGF) amino acid sequence variants. The preferred variants are resistant to proteolytic cleavage by enzymes capable of *in vivo* conversion of HGF into its two-chain form and/or contain a mutation within the protease domain of HGF.

HEPATOCYTE GROWTH FACTOR VARIANTS

BACKGROUND OF THE INVENTION

I. Field of the Invention

5   The present invention concerns amino acid sequence variants of hepatocyte growth factor (HGF), methods and means for preparing such variants, and pharmaceutical compositions comprising them.

II.   Description of Background and Related Art

10   HGF was identified initially as a mitogen for hepatocytes [Michalopoulos et al., Cancer Res.44, 4414-4419 (1984); Russel et al., J. Cell. Physiol. 119, 183-192 (1984) and Nakamura et al., Biochem. Biophys. Res. Comm. 122:1450-1459 (1984)]. Nakamura et al., Supra reported the purification of HGF from the serum of partially

15   hepatectomized rats. Subsequently, HGF was purified from rat platelets, and its subunit structure was determined [Nakamura et al., Proc. Natl. Acad. Sci. USA, 83, 6489-6493 (1986); and Nakamura et al., FEBS Letters 224, 311-316 (1987)]. The purification of human HGF (huHGF) from human plasma was first described by Gohda et al., J.

20   Clin. Invest. 81, 414-419 (1988).

        Both rat HGF and huHGF have been molecularly cloned, including the cloning and sequencing of a naturally occurring variant lacking 5 amino acids designated "delta5 HGF" [Miyazawa et al., Biochem. Biophys. Res. Comm. 163, 967-973 (1989); Nakamura et al., Nature 342,

25   440-443 (1989); Seki et al, Biochem. and Biophys. Res. Commun. 172, 321-327 (1990); Tashiro et al., Proc. Natl. Acad. Sci. USA 87, 3200-3204 (1990); Okajima et al., Eur. J. Biochem. 193, 375-381 (1990)].

        The mature form of huHGF, corresponding to the major form purified from human serum, is a disulfide linked heterodimer derived

30   by proteolytic cleavage of the human pro-hormone between amino acids R494 and V495. This cleavage process generates a molecule composed of an α-subunit of 440 amino acids (M_r 69 kDa) and a β-subunit of 234 amino acids (M_r 34 kDa). The nucleotide sequence of the hHGF cDNA reveals that both the α- and the β-chains are contained in a single

35   open reading frame coding for a pre-pro precursor protein. In the predicted primary structure of mature hHGF, an interchain S-S bridge is formed between Cys 487 of the α-chain and Cys 604 in the β-chain (see Nakamura et al., Nature, supra). The N-terminus of the α-chain

is preceded by 54 amino acids, starting with a methionine group. This segment includes a characteristic hydrophobic leader (signal) sequence of 31 residues and the prosequence. The α-chain starts at amino acid (aa) 55, and contains four Kringle domains. The so called "hairpin

5　　domain" includes amino acid residues 70-96 of wild-type human HGF. The Kringle 1 domain extends from about aa 128 to about aa 206, the Kringle 2 domain is between about aa 211 and about aa 288, the Kringle 3 domain is defined as extending from about aa 303 to about aa 383, and the Kringle 4 domain extends from about aa 391 to about aa 464 of

10　　the α-chain. It will be understood that the definition of the various Kringle domains is based on their homology with kringle-like domains of other proteins (prothrombin, plasminogen), therefore, the above limits are only approximate. As yet, the function of these Kringles has not been determined. The β-chain of huHGF shows high homology to

15　　the catalytic domain of serine proteases (38% homology to the plasminogen serine protease domain). However, two of the three residues which form the catalytic triad of serine proteases are not conserved in huHGF. Therefore, despite its serine protease-like domain, hHGF appears to have no proteolytic activity and the precise

20　　role of the β-chain remains unknown. HGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the α-chain and at positions 566 and 653 of the β-chain.

In a portion of cDNA isolated from human leukocytes in-frame deletion of 15 base pairs was observed. Transient expression of the

25　　cDNA sequence in COS-1 cells revealed that the encoded HGF molecule (delta5 HGF) lacking 5 amino acids in the Kringle 1 domain was fully functional (Seki et al., supra).

A naturally occurring huHGF variant has recently been identified which corresponds to an alternative spliced form of the huHGF

30　　transcript containing the coding sequences for the N-terminal finger and first two kringle domains of mature huHGF [Chan et al., Science 254, 1382-1385 (1991); Miyazawa et al., Eur. J. Biochem. 197, 15-22 (1991)]. This variant, designated HGF/NK2, has been proposed to be a competitive antagonist of mature huHGF.

35　　The comparison of the amino acid sequence of rat HGF with that of huHGF revealed that the two sequences are highly conserved and have the same characteristic structural features. The length of the four Kringle domains in rat HGF is exactly the same as in huHGF.

Furthermore, the cysteine residues are located in exactly the same positions; an indication of similar three-dimensional structures (Okajima et al., supra; Tashiro et al., supra).

5   The HGF receptor has been identified as the product of the c-Met proto-oncogene [Bottaro et al., Science 251, 802-804 (1991); Naldini et al., Oncogene 6, 501-504 (1991)], an 190-kDa heterodimeric (a disulfide-linked 50-kDa α-chain and a 145-kDa β-chain) membrane-spanning tyrosine kinase protein [Park et al., Proc. Natl. Acad. Sci. USA 84, 6379-6383 (1987)]. The c-Met protein becomes phosphorylated on
10   tyrosine residues of the 145-kDa β-subunit upon HGF binding.

The levels of HGF increase in the plasma of patients with hepatic failure (Gohda et al., supra) and in the plasma [Lindroos et al., Hepatol. 13, 734-750 (1991)] or serum [Asami et al., J. Biochem. 109, 8-13 (1991)] of animals with experimentally induced liver damage. The
15   kinetics of this response is rapid, and precedes the first round of DNA synthesis during liver regeneration suggesting that HGF may play a key role in initiating this process. More recently, HGF has been shown to be a mitogen for a variety of cell types including melanocytes, renal tubular cells, keratinocytes, certain endothelial
20   cells and cells of epithelial origin [Matsumoto et al., Biochem. Biophys. Res. Commun. 176, 45-51 (1991); Igawa et al., Biochem. Biophys. Res. Commun. 174, 831-838 (1991); Han et al., Biochem. 30, 9768-9780 (1991); Rubin et al., Proc. Natl. Acad. Sci. USA 88, 415-419 (1991)]. Interestingly, HGF can also act as a "scatter factor", an
25   activity that promotes the dissociation of epithelial and vascular endothelial cells in vitro [Stoker et al., Nature 327, 239-242 (1987); Weidner et al., J. Cell Biol. 111, 2097-2108 (1990); Naldini et al., EMBO J. 1, 2867-2878 (1991)]. Moreover, HGF has recently been described as an epithelial morphogen [Montesano et al., Cell 67, 901-
30   908 (1991)]. Therefore, HGF has been postulated to be important in tumor invasion and in embryonic development. Chronic c-Met/HGF receptor activation has been observed in certain malignancies [Cooper et al., EMBO J. 5, 2623 (1986); Giordano et al., Nature 339, 155 (1989)].

35   It would be desirable to better understand the structure-activity relationship of HGF in order to identify functionally important domains in the HGF amino acid sequence.

3

It would be particularly desirable to identify the amino acid residues which are responsible for the interaction of HGF with its receptor.

It would be also desirable to identify the amino acid residues which are responsible for HGF biological activity.

It would further be desirable to provide amino acid sequence variants of HGF that have altered (preferably enhanced) receptor binding affinity as compared to the corresponding mature, wild-type HGF.

It would also be desirable to provide HGF amino acid sequence variants which have retained or enhanced receptor binding affinity as compared to the corresponding wild-type HGF, but are substantially devoid of HGF biological activity. Such molecules could act as competitive antagonists of HGF action.

It would further be desirable to provide HGF amino acid sequence variants that have retained or enhanced receptor binding affinity and increased biological activity as compared to the corresponding wild-type HGF (HGF agonists). Accordingly, it is an object of the present invention to provide HGF variants having retained or improved the receptor binding affinity of the corresponding mature wild-type HGF. It is another object of the invention to provide HGF variants that have retained substantially full receptor binding affinity of the corresponding mature wild-type HGF and are substantially incapable of HGF receptor activation. It is a further object to provide HGF variants that have retained substantially full receptor binding affinity of the corresponding mature wild-type HGF and have improved biological properties.

These and further objects will be apparent to one of ordinary skill in the art.

SUMMARY OF THE INVENTION

The foregoing objects are achieved by the provision of HGF variants having amino acid alterations within various domains of the wild-type HGF amino acid sequence.

In one aspect, HGF variants are provided that are resistant to proteolytic cleavage by enzymes that are capable of _in vivo_ conversion of HGF into its two-chain form. The variants are preferably stabilized in single-chain form by site directed mutagenesis within a

region recognized by an enzyme capable of converting HGF into its two-chain form.

In a particular embodiment, such variants have an amino acid alteration at or adjacent to amino acid positions 493, 494, 495 or 496 of the wild-type huHGF amino acid sequence. The alteration preferably is the substitution of at least one amino acid at amino acid positions 493-496 of the wild-type huHGF amino acid sequence.

In another embodiment, the variants retain substantially full receptor binding affinity of the corresponding wild-type HGF and are substantially incapable of HGF receptor activation. HGF variants with enhanced receptor binding affinity and substantially lacking the ability to activate the HGF receptor are particularly preferred. Such compounds are competitive antagonists of the corresponding wild-type HGF and, when present in sufficient concentration, are capable of inhibiting the binding of their wild-type counterparts to their ligands.

In another aspect, here are provided HGF variants having an amino acid alteration at a site within the protease domain of HGF and retaining substantially full receptor binding affinity of the corresponding wild-type HGF.

In a specific embodiment, these variants have substantially retained or improved receptor binding affinity as compared to the corresponding wild-type HGF, and are substantially devoid of HGF biological activity. Such compounds, if present in sufficient concentration, will act as competitive antagonists of HGF action.

In another specific embodiment, the variants combine substantially retained or improved receptor binding affinity with improved biological activity, as compared to the corresponding wild-type HGF. Such variants are valuable as HGF agonists.

In a preferred embodiment, the HGF variants within this group comprise an alteration in a region corresponding to the catalytic site of serine proteases. More preferably the alteration is at or adjacent to any of positions 534, 673 and 692 of the wild-type human HGF (huHGF) amino acid sequence.

The alteration preferably is substitution.

In a particularly preferred embodiment, at least two of the residues at amino acid positions 534, 673 and 692 of the wild-type huHGF sequence are replaced by another amino acid.

In a preferred group of the HGF variants herein, both tyrosine
(Y) at position 673 and valine (V) at position 692 of the huHGF
sequence are replaced by another amino acid.   This alteration
potentially yields HGF variants which substantially retain the
5   receptor binding affinity of wild-type huHGF but are substantially
devoid of HGF biological activity.

The mutations around the one-chain to two-chain cleavage site and
within the protease domain may be advantageously combined for improved
biological properties.

10   Variants with increased receptor binding affinity as compared to
the corresponding wild-type HGF are particularly preferred.   The
increase in receptor binding affinity may, for example, be
accomplished by an alteration in the receptor-binding domain of the
wild-type HGF amino acid sequence, and preferably within the Kringle 1
15   domain.

Kringle 1 variants with amino acid alterations within the patch
defined by amino acid positions 159, 161, 195 and 197, or at amino
acid position 173 of the wild-type huHGF amino acid sequence are
particularly preferred, but other positions within the Kringle 1
20   domain have also been identified as having a genuine effect on the
receptor binding properties and/or the specific activity of HGF.

Furthermore, amino acid sequence variants with alterations at
amino acid positions preceding the Kringle 1 domain, in particular
those just N- or C-terminal to the hairpin domain, have been found to
25   have significantly different binding properties and biological
activity from those of the corresponding wild-type HGF.

The variants of this invention may be devoid of functional
Kringle 2 and/or Kringle 3 and/or Kringle 4 domains.

In all embodiments, huHGF amino acid sequence variants are
30   preferred.

In other embodiments, the invention relates to DNA sequences
encoding the variants described above, replicable expression   vectors
containing and capable of expressing such DNA sequences in a
transformed host cell, transformed host cells, and a process
35   comprising culturing the host cells so as to express the DNAs encoding
the HGF variants.

In yet another embodiment, the invention relates to therapeutic compositions comprising HGF variants having HGF agonist or antagonist properties.

<div align="center">BRIEF DESCRIPTION OF THE DRAWINGS</div>

5   Figure 1 is a schematic representation of the α- and β-subunits of huHGF. Shown in the α-chain are the signal sequence (boxed region) which encompasses amino acids 1 - 31, the predicted finger and four Kringle-domains, each with their respective three disulfide bonds. The cleavage site for generation of the heterodimeric α/β form of huHGF

10   immediately follows the P1 cleavage residue R494. This last residue has been specifically substituted with either E, D or A to generate HGF single-chain variants. The β-chain, which follows the cleavage site, contains homology to serine proteases. It is proposed that the α- and β-chains are held together by a unique disulfide-bridge between

15   C487(α) and C604(β) (Nakamura et al., 1989, supra). Three residues within the β-chain have been substituted individually or in combination to reconstitute the authentic residues of a serine-protease. Schematic representations of the mature forms of the C-terminal truncation variants are depicted below: N-207, deleted after

20   the first Kringle; N-303, deleted after the second Kringle; N-384, deleted after the third Kringle and the α-chain. Also shown are the variants where deletions of each of the Kringles (ΔK1, ΔK2, ΔK3 and ΔK4) were introduced. In each case, the deletions specifically remove the entire Kringle from C1 to C6.

25   Figure 2 shows the results of Western blot of wild-type rhuHGF and single-chain variants. Conditioned media from mock transfected 293 cells or stable 293 cells expressing either wild-type rhuHGF (WT) or the variants R494E, R494A or R494D were fractionated under reducing conditions on an 8% sodium-dodecyl sulfate-polyacrylamide gel and

30   blotted. The blot was reacted with polyclonal anti-HGF antisera which recognizes epitopes primarily in the α-chain. Molecular masses (kilodaltons) of the marker are as indicated. Also indicated are the positions of the α-chain and uncleaved single-chain forms of huHGF. Note that the polyclonal antibody cross-reacts with an unidentified

35   band (*) present even in the control transfected 293 cells, which do not express detectable quantities of huHGF.

       Figure 3: Mitogenic activity (A) and competitive receptor binding (B) of wild-type (WT) rhuHGF and single-chain variants. (A) Biological

<div align="center">7</div>

activity was determined by the ability of WT rhuHGF and variants to
induce DNA synthesis of rat hepatocytes in primary culture as
described in Example 2. Shown are the mean cpm from duplicates in a
representative assay. Mock supernatant from control cells did not
5   stimulate DNA synthesis in these cells (no cpm increase above
background levels). (B) To perform competitive binding, various
dilutions of supernatants of human 293 cells containing wt rhuHGF or
variants were incubated with 50 pM of the huHGF receptor-IgG fusion
protein as described in Example 2. Data represent inhibition of
10   binding as the percentage of any competing ligand from a
representative experiment and were corrected by subtraction of
background values from control 293 cells.

Figure 4: Western blot of ligand-induced tyrosine-phosphorylation
on the 145 kDa β-subunit of the HGF receptor by wild-type rhuHGF,
15   single-chain or protease domain huHGF variants. Lysates from A549
cells incubated for 5 minutes without (-) or with 200 ng/mL of
purified wt rhuHGF (WT), single-chain (R494E) or double protease
variants (Y673S,V692S) were prepared and immunoprecipitated with an
anti-HGF receptor antibody and blotted with anti-phosphotyrosine
20   antibodies. Molecular masses (kilodaltons) are as indicated.

Figure 5 depicts the nucleotide sequence encoding the plasmid
pRKS.1 (SEQ. ID. NO: 1).

Figure 6 depicts the nucleotide sequence encoding the plasmid
p.CIS.EBON (SEQ. ID. NO: 15).

25   DETAILED DESCRIPTION OF THE INVENTION
I.   Definitions

As used herein, the terms "hepatocyte growth factor", "HGF" and
"huHGF" refer to a (human) growth factor capable of specific binding
to a receptor of wild-type (human) HGF, which growth factor typically
30   has a structure with six domains (finger, Kringle 1, Kringle 2,
Kringle 3, Kringle 4 and serine protease domains), but nonetheless may
have fewer domains or may have some of its domains repeated if it
still retains its qualitative HGF receptor binding ability. This
definition specifically includes the delta5 huHGF as disclosed by Seki
35   et al., supra. The terms "hepatocyte growth factor" and "HGF" also
include hepatocyte growth factor from any non-human animal species,
and in particular rat HGF.

The terms "wild-type human hepatocyte growth factor", "native human hepatocyte growth factor", "wild-type huHGF", and "native huHGF" refer to native sequence human HGF, i.e., that encoded by the cDNA sequence published by Miyazawa, et al. 1989, supra, or Nakamura et

5   al., 1989, supra, including its mature, pre, pre-pro, and pro forms, purified from natural source, chemically synthesized or recombinantly produced. The sequences reported by Miyazawa et al. and Nakamura et al. differ in 14 amino acids. The reason for the differences is not entirely clear; polymorphism or cloning artifacts are among the

10   possibilities. Both sequences are specifically encompassed by the foregoing terms as defined for the purpose of the present invention. It will be understood that natural allelic variations exist and can occur among individuals, as demonstrated by one or more amino acid differences in the amino acid sequence of each individual. Amino acid

15   positions in the variant huHGF molecules herein are indicated in accordance with the numbering of Miyazawa et al. 1989, supra.

The terms "(HGF) biological activity", "biologically active", "activity" and "active" refer to any mitogenic, motogenic or morphogenic activities exhibited by wild-type human HGF. The HGF

20   biological activity may, for example, be determined in an in vitro or in vivo assay of hepatocyte growth promotion. Adult rat hepatocytes in primary culture have been extensively used to search for factors that regulate hepatocyte proliferation. Accordingly, the mitogenic effect of an HGF variant can be conveniently determined in an assay

25   suitable for testing the ability of an HGF molecule to induce DNA synthesis of rat hepatocytes in primary cultures, such as, for example, described in Example 2. Human hepatocytes are also available from whole liver perfusion on organs deemed unacceptable for transplantation, pare-downs of adult livers used for transplantation

30   in children, fetal livers and liver remnants removed at surgery for other indications. Human hepatocytes can be cultured similarly to the methods established for preparing primary cultures of normal rat hepatocytes. Hepatocyte DNA synthesis can, for example, be assayed by measuring incorporation of [3H]thymidine into DNA, with appropriate

35   hydroxyurea controls for replicative synthesis.

The effect of HGF variants on hepatocyte growth can also be tested in vivo in animal models of liver dysfunction and regeneration, such as in rats following partial hepatectomy, or carbon tetrachloride

caused hepatic injury, in D-galactosamine induced acute liver failure
models, etc. According to a suitable protocol, a liver poison, e.g.
α-naphthylisothiocyanate (ANIT) is administered to rats in a
predetermined concentration capable of causing reproducible
5   significant elevation of liver enzyme and bilirubin levels. The rats
are then treated with the HGF variant to be tested, sacrificed and the
liver enzyme and bilirubin levels are determined. The livers are
additionally observed for hepatic lesions.

The expression "retaining substantially full receptor binding
10   affinity of wild-type (hu)HGF" and grammatical variant thereof as used
herein mean that the receptor binding affinity of the HGF variant is
not less then about 70%; preferably not less than about 80%, more
preferably not less than about 90%, most preferably not less than
about 95% of the affinity with which wild-type (hu)HGF binds its
15   native receptor.

The terms "substantially incapable of HGF receptor activation"
and "substantially devoid of HGF biological activity" mean that the
activity exhibited by an HGF variant is less than about 20%,
preferably less than about 15%, more preferably less than about 10%,
20   most preferably less than about 5% of the respective activity of wild-
type (human) HGF in an established assay of receptor activation or HGF
biological activity, as hereinabove defined.

The terms "amino acid" and "amino acids" refer to all naturally
occurring L-α-amino acids. This definition is meant to include
25   norleucine, ornithine, and homocysteine. The amino acids are
identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan
Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

(line numbers 30 and 35 appear beside the Glu and Val rows respectively)

These amino acids may be classified according to the chemical
composition and properties of their side chains. They are broadly

classified into two groups, charged and uncharged. Each of these groups is divided into subgroups to classify the amino acids more accurately:

I. Charged Amino Acids

5   Acidic Residues: aspartic acid, glutamic acid

Basic Residues: lysine, arginine, histidine

II. Uncharged Amino Acids

Hydrophilic Residues: serine, threonine, asparagine, glutamine

10   Aliphatic Residues: glycine, alanine, valine, leucine, isoleucine

Non-polar Residues: cysteine, methionine, proline

Aromatic Residues: phenylalanine, tyrosine, tryptophan

The terms "alteration", "amino acid alteration", "variant" and

15   "amino acid sequence variant" refer to HGF molecules with some differences in their amino acid sequences as compared to wild-type (human) HGF. Ordinarily, the variants will possess at least about 80% homology with those domains of wild-type (human) HGF that are retained in their structure, and preferably, they will be at least about 90%

20   homologous with such domains.

Substitutional HGF variants are those that have at least one amino acid residue in the corresponding wild-type HGF sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the

25   molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Substantial changes in the activity of the HGF molecule may be obtained by substituting an amino acid with a side chain that is significantly different in charge and/or structure from that of the

30   native amino acid. This type of substitution would be expected to affect the structure of the polypeptide backbone and/or the charge or hydrophobicity of the molecule in the area of the substitution.

Moderate changes in the activity of the HGF molecule would be expected by substituting an amino acid with a side chain that is

35   similar in charge and/or structure to that of the native molecule. This type of substitution, referred to as a conservative substitution, would not be expected to substantially alter either the structure of

the polypeptide backbone or the charge or hydrophobicity of the molecule in the area of the substitution.

Insertional HGF variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular

5   position in the wild-type HGF molecule. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid. The insertion may be one or more amino acids. Ordinarily, the insertion will consist of one or two conservative amino acids. Amino acids similar in charge and/or

10 structure to the amino acids adjacent to the site of insertion are defined as conservative. Alternatively, this invention includes insertion of an amino acid with a charge and/or structure that is substantially different from the amino acids adjacent to the site of insertion.

15 Deletional variants are those with one or more amino acids in the wild-type HGF molecule removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the HGF molecule.

The notations used throughout this application to describe huHGF

20 amino acid sequence variants are described below. The location of a particular amino acid in the polypeptide chain of huHGF is identified by a number. The number refers to the amino acid position in the amino acid sequence of the mature, wild-type human HGF polypeptide as disclosed in Miyazawa et al., 1989, supra. In the present application,

25 similarly positioned residues in huHGF variants are designated by these numbers even though the actual residue number is not so numbered due to deletions or insertions in the molecule. This will occur, for example, with site-directed deletional or insertional variants. The amino acids are identified using the one-letter code. Substituted

30 amino acids are designated by identifying the wild-type amino acid on the left side of the number denoting the position in the polypeptide chain of that amino acid, and identifying the substituted amino acid on the right side of the number.

For example, replacement of the amino acid arginine (R) by

35 glutamic acid (E) at amino acid position 494 of the wild-type huHGF molecule yields a huHGF variant designated R494E huHGF. Similarly, the huHGF variant obtained by substitution of serine (S) for tyrosine (Y) at amino acid position 673 and serine (S) for valine (V) at amino

acid position 692 of the wild-type huHGF molecule is designated
Y673S,V692S huHGF.

Deletional variants are identified by indicating the amino acid
residue and position at either end of the deletion, inclusive, and
5   placing the Greek letter delta, "Δ", to the left of the indicated
amino acids. Deletion of a single amino acid is indicated by placing
Δ to the left of the single letter code and number indicating the
position of the deleted amino acid.

Insertional variants are designated by the use of brackets "[]"
10   around the inserted amino acids, and the location of the insertion is
denoted by indicating the position of the amino acid on either side of
the insertion.

The alterations in the amino acid sequence of the HGF variants
herein are indicated with reference to amino acid positions in the
15   wild-type human HGF amino acid sequence. (Miyazawa et al., supra).
Methods for the alignment of homologous amino acid sequences from
various species are well known in the art.

The terms "DNA sequence encoding", "DNA encoding" and "nucleic
acid encoding" refer to the order or sequence of deoxyribonucleotides
20   along a strand of deoxyribonucleic acid. The order of these
deoxyribonucleotides determines the order of amino acids along the
polypeptide chain. The DNA sequence thus codes for the amino acid
sequence.

The terms "replicable expression vector" and "expression vector"
25   refer to a piece of DNA, usually double-stranded, which may have
inserted into it a piece of foreign DNA. Foreign DNA is defined as
heterologous DNA, which is DNA not naturally found in the host cell.
The vector is used to transport the foreign or heterologous DNA into a
suitable host cell. Once in the host cell, the vector can replicate
30   independently of the host chromosomal DNA, and several copies of the
vector and its inserted (foreign) DNA may be generated. In addition,
the vector contains the necessary elements that permit translating the
foreign DNA into a polypeptide. Many molecules of the polypeptide
encoded by the foreign DNA can thus be rapidly synthesized.

35   In the context of the present invention the expressions "cell",
"cell line", and "cell culture" are used interchangeably, and all such
designations include progeny.

The terms "transformed (host) cell" , "transformant" and "transformed" refer to the introduction of DNA into a cell. The cell is termed a "host cell". The introduced DNA is usually in the form of a vector containing an inserted piece of DNA. The introduced DNA

5 sequence may be from the same species as the host cell or a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign and some homologous DNA. The words transformants and transformed (host) cells include the primary subject cell and cultures derived therefrom, without regard to the number of

10 transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological property as screened for in the originally transformed cell are included.

15   The technique of "polymerase chain reaction" or "PCR", as used herein, generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195, issued 28 July 1987 and in Current Protocols in Molecular Biology, Ausubel et al. eds., Greene

20 Publishing Associates and Wiley-Interscience 1991, Volume 2, Chapter 15.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population

25 are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. The monoclonal antibodies include hybrid and recombinant antibodies produced by splicing a variable (including

30 hypervariable) domain of an anti-selectin ligand antibody with a constant domain (e.g. "humanized" antibodies), only one of which is directed against a selectin, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or

35 immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')$_2$, and Fv). Cabilly, et al., U.S. Pat. No. 4,816,567; Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New York,

1987). Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from such a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

5   The term "immunoglobulin" generally refers to polypeptides comprising a light or heavy chain usually both disulfide bonded in the native "Y" configuration, although other linkage between them, including tetramers or aggregates thereof, is within the scope hereof.

10   Immunoglobulins (Ig) and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner et al., Nature 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler et al., Proc. Nat'l. Acad. Sci. USA 77:2197 (1980); Raso et
15   al., Cancer Res. 41:2073 (1981); Morrison et al., Ann. Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison et al., Proc. Nat'l. Acad. Sci. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and
20   references cited therein. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG_1, IgG_2, IgG_3, or IgG_4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG_1 or IgG_3.

II.   Selection of the HGF Variants

25   The present invention is based upon the study of structure-activity and structure-receptor binding relationship in amino acid sequence variants of HGF.

Certain HGF variants of the present invention are resistant to proteolytic cleavage by enzymes that are capable of in vivo conversion
30   of the single-chain HGF proenzyme into its two-chain form. Such enzymes are trypsin-like proteases. Absent alterations, the proteolytic cleavage takes place between Arg494 and Val495 of the wild-type huHGF sequence. The resistance to proteolytic cleavage is preferably achieved by site-directed mutagenesis within a region
35   recognized by an enzyme capable of converting HGF into its two-chain form, and preferably within the Leu-Arg-Val-Val (LRVV) sequence at amino acid residues 493-496 of the wild-type huHGF sequence. The variants herein may, for example, contain single or multiple amino

acid substitutions, insertions or deletions at or adjacent to amino acid positions 493, 494, 495 and 496 in the wild-type human HGF amino acid sequence.

A preferred alteration is the replacement of arginine at amino
5 acid position 494 with any other amino acid, preferably glutamic acid, aspartic acid or alanine. In general, the substitution of smaller, apolar or acidic amino acids for arginine at this position is believed to yield single-chain HGF variants.

Alternatively or in addition, the replacement of valine at
10 position 495 by another amino acid is expected to block the one-chain to two-chain cleavage. Bulkier amino acids, such as tyrosine, phenylalanine, etc. are preferred for substitution at this position.

Other HGF variants of the present invention are altered at a site within the protease domain of HGF and retain substantially full
15 receptor binding affinity of the corresponding (preferably human) wild-type HGF. The protease domain follows the cleavage site between amino acid positions 494 and 495 in the wild-type huHGF sequence, and shows a high degree of homology with the catalytic domain of known serine proteases. The conservation does not apply to
20 the active site of serine proteases. In human plasmin, which is formed from its proenzyme, plasminogen, residues His-42, Asp-85 and Ser-181 form the catalytic site (catalytic triad). This catalytic triad is highly conserved in serine proteases. In the huHGF amino acid sequence asparagine is retained at amino acid position 594,
25 however, position 534 (corresponding to position 42 of plasmin) is occupied by glutamine instead of histidine, and position 673 (corresponding to position 181 of plasmin) by tyrosine instead of serine. A preferred group of the protease domain alterations herein involves one or both of amino acid positions 673 and 534.
30 Alternatively, or in addition, the alteration may be at position 692 of the huHGF amino acid sequence. In all instances, the alteration preferably is the substitution of one or more different amino acids for the residues at these positions of the native huHGF amino acid sequence.

35   Tyrosine at amino acid position 673 is preferably replaced by an amino acid which has no bulky aromatic or heterocyclic moieties. Such amino acids include serine, threonine, asparagine, cysteine, glycine,

alanine and valine. In the preferred variants, serine is substituted for tyrosine at this position.

Valine at amino acid position 692 preferably is substituted by a polar amino acid, such as serine, threonine, asparagine or glutamine, preferably serine.

In a preferred group of the HGF amino acid sequence variants herein, both position 673 and position 692 are substituted by one of the foregoing amino acids, preferably serine. Such variants may additionally contain an alteration (preferably substitution) at amino acid position 534. The latter alteration may be the substitution of histidine for glutamine in the wild-type huHGF amino acid sequence.

The single, double or triple mutations within the protease domain may be combined with additional alterations in the wild-type HGF amino acid sequence. Such further alterations may, for example, be at or around the one-chain to two chain cleavage site of the HGF molecule, as hereinabove described, and may result in variants which are substantially in single-chain form.   —

Additional alterations may be at the C-terminal end and/or in the Kringle·domains of the HGF molecule. In addition to the deletion mutants disclosed in the examples, HGF variants with alterations within the Kringle 1 domain are of great interest. As we have found that the receptor binding domain is contained within the finger and the Kringle 1 regions of the HGF molecule, amino acid alterations within these domains are expected to significantly alter the receptor binding·properties (and the biological activity) of the variants of the present invention. Alterations at residues that are most exposed to the interior in the Kringle structure (mostly charged residues) are particularly likely to cause profound changes in the receptor binding properties and/or biological activity of the HGF variants.

Alterations within the Kringle 1 domain preferably are within the patch defined by amino acid positions 159, 161, 195 and 197 of the wild-type huHGF amino acid sequence or at corresponding positions in a non-human HGF amino acid sequence. Another preferred site for amino acid alteration is at position 173 of the wild-type huHGF amino acid sequence. The latter position is at the opposite side as compared to the surface defined by amino acid position 159, 161, 195 and 197 and the reasons for its involvement in the binding properties and biological activity of HGF have not yet been fully identified.

Some illustrative huHGF variants within the scope herein are as follows: R494E; R494D; R494A; V495Y; V495F; R494E, V495Y; R494E, V495F; R494D, V495Y; R494D, V495F; R494A, V495Y; R494A, V495F; R494[E]V495; R494[D]V495; R494[A]V495; R494[Y]V495; R494[F]V495;

5   R494E, Q534H; R494E, Y673S; R494E, V692S; R494D, Q534H; R494D, Y673S; R494D, V692S, R494A, Q534H; R494A, V673S; R494A, V692S, R494E, Y673S, V692S; R494D, Y673S, V692S, R494A, Y673S, V692S, R494E, Q534H, Y673S, V692S; R494D, Q534H, Y673S, V692S; R494A, Q534H, Y673S, V692S; E159A; S161A; F162A, L163A, S165A, S166A; F162A; L163A, S165A, S166A; Y167F;

10   Y167A; R168A; Q173A; Q173A, E174A, N175A; N193A; R195A; R197A; N193A, E195A, R197A; K52A; D54A; K52A, D54A; H114A; H114A, E115A, D117A; E115A; D117A; variants with combinations of any of the foregoing alterations; ΔK3 and/or ΔK4 variants comprising any of the foregoing alterations; corresponding delta5-huHGF variants and non-human animal

15   HGF variants.

III. Construction of the HGF Variants

Whereas any technique known in the art can be used to perform site-directed mutagenesis, e.g. as disclosed in Sambrook et al.

20   [Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, New York (1989)], oligonucleotide-directed mutagenesis is the preferred method for preparing the HGF variants of this invention. This method, which is well known in the art [Adelman et al. DNA, 2:183 (1983), Sambrook et al., Supra], is particularly

25   suitable for making substitution variants, it may also be used to conveniently prepare deletion and insertion variants.

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed

30   mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam (1981). These phage are readily commercially available and their use is generally well known to those skilled in the art.

35   Alternatively, plasmid vectors that contain a single-stranded phage origin of replication (Veira et al., Meth. Enzymol., 153: 3 (1987)) may be employed to obtain single-stranded DNA.

The oligonucleotides are readily synthesized using techniques well known in the art such as that described by Crea et al. (_Proc. Nat'l. Acad. Sci. USA_, _75_:5765 [1978]).

5   The specific mutagenesis method followed in making the HGF variants of Example 1 was described by Kunkel _et al._, _Methods in Enzymol._ _154_ 367-382 (1987).

Mutants with more than one amino acid substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated
10   simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If however, the amino acids are located some distance from each other (separated by more than ten amino acids, for example) it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one
15   of two alternative methods may be employed. In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired
20   amino acid substitutions. The alternative method involves two or more rounds of mutagenesis to produce the desired mutant.

Another method for making mutations in the DNA sequence encoding wild-type HGF or a variant molecule known in the art, involves cleaving the DNA sequence encoding the starting HGF molecule at the
25   appropriate position by digestion with restriction enzymes, recovering the properly cleaved DNA, synthesizing an oligonucleotide encoding the desired amino acid sequence and flanking regions such as polylinkers with blunt ends (or, instead of polylinkers, digesting the synthetic oligonucleotide with the restriction enzymes also used to cleave the
30   HGF encoding DNA, thereby creating cohesive termini), and ligating the synthetic DNA into the remainder of the HGF encoding structural gene.

PCR mutagenesis is also suitable for making the HGF variants of the present invention, for example, as described in U.S. Patent No. 4,683,195 issued 28 July 1987 and in _Current Protocols in Molecular_
35   _Biology_, Ausubel _et al._, eds. Greene Publishing Associates and Wiley-Interscience, Volume 2, Chapter 15, 1991. While the following discussion refers to DNA, it is understood that the technique also find application with RNA. The PCR technique generally refers to the

following procedure. When small amounts of template DNA are used as
starting material in a PCR, primers that differ slightly in sequence
from the corresponding region in a template DNA can be used to
generate relatively large quantities of a specific DNA fragment that

5   differs from the template sequence only at the positions where the
primers differ from the template. For introduction of a mutation into
a plasmid DNA, one of the primers is designed to overlap the position
of the mutation and to contain the mutation; the sequence of the other
primer must be identical to a stretch of sequence of the opposite

10   strand of the plasmid, but this sequence can be located anywhere along
the plasmid DNA. It is preferred, however, that the sequence of the
second primer is located within 200 nucleotides from that of the
first, such that in the end the entire amplified region of DNA bounded
by the primers can be easily sequenced. PCR amplification using a

15   primer pair like the one just described results in a population of DNA
fragments that differ at the position of the mutation specified by the
primer, and possibly at other positions, as template copying is
somewhat error-prone. If the ratio of template to product material is
extremely low, the vast majority of product DNA fragments incorporate

20   the desired mutation(s). This product material is used to replace the
corresponding region in the plasmid that served as PCR template using
standard DNA technology. Mutations at separate positions can be
introduced simultaneously by either using a mutant second primer or
performing a second PCR with different mutant primers and ligating the

25   two resulting PCR fragments simultaneously to the vector fragment in a
three (or more)-part ligation.

       The cDNA encoding the HGF variants of the present invention is
inserted into a replicable vector for further cloning or expression.

       Suitable vectors are prepared using standard recombinant DNA

30   procedures. Isolated plasmids and DNA fragments are cleaved,
tailored, and ligated together in a specific order to generate the
desired vectors.

       After ligation, the vector with the foreign gene now inserted is
transformed into a suitable host cell. The transformed cells are

35   selected by growth on an antibiotic, commonly tetracycline (tet) or
ampicillin (amp), to which they are rendered resistant due to the
presence of tet and/or amp resistance genes on the vector. If the
ligation mixture has been transformed into a eukaryotic host cell,

transformed cells may be selected by the DHFR/MTX system. The transformed cells are grown in culture and the plasmid DNA (plasmid refers to the vector ligated to the foreign gene of interest) is then isolated. This plasmid DNA is then analyzed by restriction mapping
5   and/or DNA sequencing. DNA sequencing is generally performed by either the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods of Enzymology, 65:499 (1980).

Prokaryotes are the preferred host cells for the initial cloning
10   steps of this invention. They are particularly useful for rapid production of large amounts of DNA, for production of single-stranded DNA templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for DNA sequencing of the mutants generated. For expressing the HGF variants of the present invention
15   eukaryotic hosts, such as eukaryotic microbes (yeast) and multicellular organisms (mammalian cell cultures) may also be used. Examples of prokaryotes, e.g. E. coli, eukaryotic microorganisms and multicellular cell cultures, and expression vectors, suitable for use in producing the HGF variants of the present invention are, for
20   example, those disclosed in WO 90/02798 (published 22 March 1990).

Cloning and expression methodologies are well known in the art and are, for example, disclosed in the foregoing published PCT patent application (WO 90/02798).

If mammalian cells are used as host cells, transfection generally
25   is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, Virology, 52: 546 (1978). However, other methods for introducing DNA into cells such as nuclear injection, electroporation, or protoplast fusion are also suitably used.

30   If yeast are used as the host, transfection is generally accomplished using polyethylene glycol, as taught by Hinnen, Proc. Natl. Acad. Sci. U.S.A., 75: 1929-1933 (1978).

If prokaryotic cells or cells that contain substantial cell wall constructions are used, the preferred method of transfection is
35   calcium treatment using calcium as described by Cohen et al., Proc. Natl. Acad. Sci. (USA) 69: 2110 (1972), or more recently electroporation.

The HGF variant preferably is recovered from the culture medium as a secreted protein, although it also may be recovered from host cell lysates when directly expressed without a secretory signal. When the variant is expressed in a recombinant cell other than one of human

5   origin, the variant is thus completely free of proteins of human origin. However, it is necessary to purify the variant from recombinant cell proteins in order to obtain preparations that are substantially homogeneous as to protein. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris.

10   The variant is then purified from contaminant soluble proteins, for example, by an appropriate combination of conventional chromatography methods, e.g. gel filtration, ion-exchange, hydrophobic interaction, affinity, immunoaffinity chromatography, reverse phase HPLC; precipitation, e.g. ethanol precipitation, ammonium sulfate

15   precipitation, or, preferably, immunoprecipitation with anti-HGF (polyclonal or monoclonal) antibodies covalently linked to Sepharose. Due to its high affinity to heparine, HGF can be conveniently purified on a heparin, such as heparine-Sepharose column. One skilled in the art will appreciate that purification methods suitable for native HGF

20   may require modification to account for changes in the character of HGF or its variants upon expression in recombinant cell culture.

As hereinabove described, huHGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the α-chain and at positions 566 and 653 of the β-chain. These positions

25   are conserved in the rat HGF amino acid sequence. Glycosylation variants are within the scope herein.

Glycosylation of polypeptides is typically either N-linked or O-linked.. N-linked refers to the attachment of the carbohydrate moiety to the side-chain of an asparagine residue. The tripeptide sequences,

30   asparagine-X-serine and asparagine-X-threonine, wherein X is any amino acid except proline, are recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid,

35   most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be involved in O-linked glycosylation. O-linked glycoslation sites may, for example, be modified by the addition of, or substitution by, one or more serine or threonine

residue to the amino acid sequence of the HGF molecule. For ease, changes are usually made at the DNA level, essentially using the techniques discussed hereinabove with respect to the amino acid sequence variants.

5   Chemical or enzymatic coupling of glycosydes to the HGF variants of the present invention may also be used to modify or increase the number or profile of carbohydrate substituents. These procedures are advantageous in that they do not require production of the polypeptide that is capable of O-linked (or N-linked) glycosylation. Depending on

10   the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free hydroxyl groups such as those of cysteine, (d) free sulfhydryl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan or (f) the amide group

15   of glutamine. These methods are described in WO 87/05330 (published 11 September 1987), and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

        Carbohydrate moieties present on an HGF variant may also be removed chemically or enzymatically. Chemical deglycosylation

20   requires exposure to trifluoromethanesulfonic acid or an equivalent compound. This treatment results in the cleavage of most or all sugars, except the linking sugar, while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al., Arch. Biochem. Biophys. 259, 52 (1987) and by Edge et al., Anal.

25   Biochem. 118, 131 (1981). Carbohydrate moieties can be removed by a variety of endo- and exoglycosidases as described by Thotakura et al., Meth. Enzymol. 138, 350 (1987). Glycosylation is suppressed by tunicamycin as described by Duskin et al., J. Biol. Chem. 257, 3105 (1982). Tunicamycin blocks the formation of protein-N-glycosydase

30   linkages.

        Glycosylation variants of the amino acid sequence variants herein can also be produced by selecting appropriate host cells. Yeast, for example, introduce glycosylation which varies significantly from that of mammalian systems. Similarly, mammalian cells having a different

35   species (e.g. hamster, murine, insect, porcine, bovine or ovine) or tissue (e.g. lung, liver, lymphoid, mesenchymal or epidermal) origin than the source of the selectin variant, are routinely screened for the ability to introduce variant glycosylation. Covalent

modifications of an HGF variant molecule are included within the scope
herein. Such modifications are traditionally introduced by reacting
targeted amino acid residues of the HGF variant with an organic
derivatizing agent that is capable of reacting with selected side-
5   chains or terminal residues, or by harnessing mechanisms of post-
translational modifications that function in selected recombinant host
cells. The resultant covalent derivatives are useful in programs
directed at identifying residues important for biological activity,
for immunoassays of the HGF variants, or for the preparation of anti-
10   HGF antibodies for immunoaffinity purification of the recombinant
glycoprotein. For example, complete inactivation of the biological
activity of the protein after reaction with ninhydrin would suggest
that at least one arginyl or lysyl residue is critical for its
activity, whereafter the individual residues which were modified under
15   the conditions selected are identified by isolation of a peptide
fragment containing the modified amino acid residue. Such
modifications are within the ordinary skill in the art and are
performed without undue experimentation.

       Derivatization with bifunctional agents is useful for preparing
20   intramolecular aggregates of the HGF variants as well as for cross-
linking the HGF variants to a water insoluble support matrix or
surface for use in assays or affinity purification. In addition, a
study of interchain cross-links will provide direct information on
conformational structure. Commonly used cross-linking agents include
25   1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-
hydroxysuccinimide esters, homobifunctional imidoesters, and
bifunctional maleimides. Derivatizing agents such as methyl-3-[(p-
azidophenyl)dithiol]propioimidate yield photoactivatable intermediates
which are capable of forming cross-links in the presence of light.
30   Alternatively, reactive water insoluble matrices such as cyanogen
bromide activated carbohydrates and the systems reactive substrates
described in U.S. patent Nos. 3,959,642; 3,969,287; 3,691,016;
4,195,128; 4,247,642; 4,229,537; 4,055,635; and 4,330,440 are employed
for protein immobilization and cross-linking.

35   Certain post-translational modifications are the result of the
action of recombinant host cells on the expressed polypeptide.
Glutaminyl and aspariginyl residues are frequently post-
translationally deamidated to the corresponding glutamyl and aspartyl

residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

5   Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)].

10   Other derivatives comprise the novel HGF variants of this invention covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e. a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or in
15   vitro methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyvinylalkylene ethers such a polyethylene glycol, polypropylene glycol.

20   The HGF variants may be linked to various nonproteinaceous polymers, such as polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The HGF variants may be entrapped in microcapsules prepared, for
25   example, by coacervation techniques or by interfacial polymerization, in colloidal drug delivery systems (e.g. liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th Edition, Osol, A., Ed. (1980).   An
30   HGF variant sequence can be linked to a immunoglobulin constant domain sequence as hereinbefore defined. The resultant molecules are commonly referred to as HGF variant-immunoglobulin chimeras. Such chimeras can be constructed essentially as described in WO 91/08298 (published 13 June 1991).

35   Ordinarily, the HGF variant is fused C-terminally to the N-terminus of the constant region of an immunoglobulin in place of the variable region(s), however N-terminal fusions of the selectin

variants are also desirable. The transmembrane regions of the HGF variants are preferably inactivated or deleted prior to fusion.

Typically, such fusions retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin

5   heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture.

10 Alternatively, however, the HGF variant-immunoglobulin chimeras of this invention may be synthesized according to known methods.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics

15 of the HGF variant.

In some embodiments, the hybrid immunoglobulins are assembled as monomers, or hetero- or homo-multimers, and particularly as dimers of tetramers, essentially as illustrated in WO 91/08298, Supra.

In a preferred embodiment, the C-terminus of a sequence which

20 contains the binding site(s) for an HGF receptor, is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. immunoglobulin G_1. It is possible to fuse the entire heavy chain constant region to the sequence containing the receptor binding

25 site(s). However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 [Kobet et al., Supra], or analogous sites of other immunoglobulins) is used in the fusion. In a particularly

30 preferred embodiment, the amino acid sequence containing the receptor binding site(s) is fused to the hinge region and C_H2 and C_H3 or C_H1, hinge, C_H2 and C_H3 domains of an IgG_1, IgG_2 or IgG_3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

35 HGF variant-immunoglobulin chimeras may, for example, be used in protein A purification, immunohistochemistry, and immunoprecipitation techniques in place of anti-HGF antibodies, and can facilitate screening of inhibitors of HGF-HGF receptor interactions.

Therapeutically, they are expected to confer advantages such as longer half-life as compared to the corresponding HGF variant molecule.

IV. Therapeutic Compositions

5   The HGF variants with enhanced receptor binding affinity can be used to block the binding of wild-type HGF to its receptor. This would permit the treatment of pathologic conditions associated with the activation of an HGF receptor, such as malignancies associated with chronic HGF receptor activation.

10   The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the HGF product is combined in admixture with a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences,

15   16th ed., 1980, Mack Publishing Co., edited by Oslo et al. These compositions will typically contain an effective amount of the HGF variant, for example, from on the order of about 0.5 to about 10 mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective

20   administration to the patient. The variants may be administered parenterally or by other methods that ensure its delivery to the bloodstream in an effective form.

Compositions particularly well suited for the clinical administration of the HGF variants used to practice this invention

25   include sterile aqueous solutions or sterile hydratable powders such as lyophilized protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the formulation to render the formulation isotonic.

Dosages and desired drug concentrations of pharmaceutical

30   compositions of this invention may vary depending on the particular use envisioned. A typical effective dose in rat experiments is about 250 μg/kg administered as an intravenous bolus injection. Interspecies scaling of dosages can be performed in a manner known in the art, e.g. as disclosed in Mordenti et al., Pharmaceut. Res. 8,

35   1351 (1991) and in the references cited therein.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

V.   Examples

A series of recombinant huHGF (rhuHGF) variants were produced to
determine the structural and functional importance of the cleavage of
the prohormone to the α/β dimer and of the Kringle and protease-like

5   domains.   Mutations were introduced into the huHGF cDNA in a CMV
based expression plasmid and conditioned media from stable populations
of human 293 cells expressing each variant were assayed by Western
blotting to monitor the size and expression level of the HGF variants.

The concentration of each huHGF derivative was confirmed with two

10   types of sandwich ELISA assays.   The differences in expression levels
found in ELISA correlated with those observed on Western blots.   For
most variants, the level of expression was in the range of 1-5 mg/mL.
For variants with expression levels below 0.6 mg/mL, the conditioned
media was concentrated.

15   The mitogenic activity on liver cells in primary culture and
ability to bind to the HGF receptor was then determined.   The extra-
cellular domain of the HGF receptor was fused to the constant region
(Fc) of an human IgG and binding was performed in solution.

The construction of the rhuHGF variants, the assay methods and

20   the analysis of the results obtained with the various mutants are
described in the following examples.

EXAMPLE 1

Recombinant Production of the huHGF Variants

25   A.   Site-directed mutagenesis

Plasmid DNA isolation, polyacrylamide and agarose gel
electrophoresis were performed as disclosed in Sambrook et al., supra.

Mammalian expression plasmid pRK 5.1 with a CMV promotor
(Genentech, Inc.) was used for mutagenesis of huHGF allowing secretion

30   of the HGF variants in the culture medium and directly assayed for
biological activity and binding.   This expression vector is a
derivative of pRK5, the construction of which is disclosed in EP
307,247 published 15 March 1989.   The nucleotide sequence encoding
this the pRK 5.1 vector is shown in Figure 5 (SEQ. ID. NO: 1).

35   The huHGF cDNA used corresponds to the 728 amino acid form as
published earlier (Miyazawa et al., 1989, supra).

Mutagenesis was performed according to the method of Kunkel using
the commercially available dut- ung- strain of E. coli   [Kunkel et

al., Method. Enzymol. 154, 367-382 (1987)]. Synthetic
oligonucleotides used for in vitro mutagenesis and sequencing primers
were prepared using the Applied Biosystem 380A DNA synthesizer as
described [Matteucci et al., J. Am. Chem. Soc. 103, 3185-3191 (1981)].

5   For generation of the desired mutants, oligonucleotides of sequences
coding for the desired amino acid substitutions were synthesized and
used as primers. The oligonucleotides were annealed to single-
stranded pRK51-huHSA that had been prepared by standard procedures
[Viera et al., Method. Enzymol. 142, 3 (1987)].

10   A mixture of three deoxyribonucleotides, deoxyriboadenosine
(dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), was
combined with a modified thio-deoxyribonuleosine called dCTP(aS)
provided in the kit by the manufacturer, and added to the single
stranded pRK 5.1-huHGF to which was annealed the oligonucleotide.

15   Upon addition of DNA polymerase to this mixture, a strand of DNA
identical to pRK 5.1-huHGF except for the mutated bases was generated.
In addition, this new strand of DNA contained dCTP(aS) instead of
dCTP, which served to protect from restriction endonuclease digestion.
After the template strand of the double-stranded heteroduplex was

20   nicked with an appropriate restriction enzyme, the template strand was
digested with ExoIII nuclease past the region that contained the
mutagenic oligomer. The reaction was then stopped to leave a molecule
hat was only partly single-stranded. A complete double-stranded DNA
homoduplex molecule was then formed by DNA polymerase in the presence

25   of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase.

The following oligonucleotides were prepared to use as primers to
generate pRK 5.1-huHGF variant molecules:

R494E huHGF: TTGGAATCCCATTTACAACCTCGAGTTGTTTCGTTTTGGCACAAGAT

(SEQ. ID. NO: 2)

30   R494D huHGF: GAATCCCATTTACGACGTCCAATTGTTTCG (SEQ. ID. NO: 3)

R494A huHGF: CCCATTTACAACTGCCAATTGTTTCG   (SEQ. ID. NO: 4)

Q534H huHGF: AGAAGGGAAACAGTGTCGTGCA   (SEQ. ID. NO: 5)

Y673S huHGF: AGTGGGCCACCAGAATCCCCCT   (SEQ. ID. NO: 6)

V692S huHGF: TCCACGACCAGGAGAAATGACAC   (SEQ. ID. NO: 7)

35   ΔK1 huHGF: GCATTCAACTTCTGAGTTTCTAATGTAGTC   (SEQ. ID. NO: 8)

ΔK2 huHGF: CATAGTATTGTCAGCTTCAACTTCTGAACA   (SEQ. ID. NO: 9)

ΔK3 huHGF: TCCATGTGACATATCTTCAGTTGTTTCCAA   (SEQ. ID. NO:10)

ΔK4 huHGF: TGTGGTATCACCTTCATCTTGTCCATGTGA   (SEQ. ID. NO:11)

N-303 huHGF: ACCTTGGATGCATTAAGTTGTTTC   (SEQ. ID. NO:12)

N-384 huHGF: TTGTCCATGTGATTAATCACAGT   (SEQ. ID. NO:13)

α-chain: GTTCGTGTTGGGATCCCATTTACCTATCGCAATTG (SEQ. ID. NO:14)

The Y673S, V692S huHGF variant was obtained from wild-type huHGF
5   as a template, using both oligonucleotides used for generating the two
mutations.

The mutant huHGF constructs generated using the protocol above
were transformed in E. coli host strain MM294tonA using the standard
calcium chloride procedure (Sambrook et al., supra) for preparation
10   and transformation of competent cells. MM294tonA (which is resistant
to T1 phage) was prepared by the insertion and subsequent imprecise
excision of a Tn10 transposon into the tonA gene. This gene was then
inserted, using transposon insertion mutagenesis [Kleckner et al., J.
Mol. Biol. 116, 125-159 (1977)], into E. coli host MM294 (ATCC
15   31,446).

The DNA extract from individual colonies of bacterial
transformants using the standard miniprep procedure of Sambrook et
al., supra. The plasmids were further purified by passage over a
Sephacryl CL6B spin column, and then analyzed by sequencing and by
20   restriction endonuclease digestion and agarose gel electrophoresis.

B.   Transfection of Human Embryonic Kidney 293 Cells

Plasmids with the correct sequence were used to transfect human
fetal kidney 293 cells by the calcium phosphate method. 293 cells were
growth to 70% confluence in 6-well plates. 2.5 μg of huHGF plasmid
25   DNA variant was dissolved in 150 μl of 1 mM Tris-HCl, 0.1 mM EDTA,
0.227 M.CaCl$_2$. Added to this (dropwise while vortexing) was 150 μl of
50 mM HEPES buffer (pH 7.35), 280 mM NaCl, 1.5 mM NaPO$_4$, and the
precipitate was allowed to form for ten minutes at 25 °C. The
suspended precipitate was then added to the cells in the individual
30   wells in a 6-well plate. The cell monolayers were incubated for 4
hours in the presence of the DNA precipitate, washed once with PBS,
and cultured in serum-free medium for 72h. When stable populations
were made, the HGF cDNA was subcloned in an episomal CMV driven
expression plasmid pCisEBON (G. Cachianes, C, Ho, R. Weber, S.
35   Williams, D. Goeddel, and D. Lueng, in preparation). pCisEBON is a
pRK5 derivative; its underlying nucleotide sequence is shown in Figure
6 (SEQ. ID. NO: 15). The populations were directly selected in
Neomycin selective medium.

EXAMPLE 2

Assay Methods

In view of the pleiotropic activities of HGF, a molecule with a
structure unlike any other known growth factor, it is important to
5   understand the molecular interaction of this factor with its receptor.
The huHGF variants produced as described in Example 1 were analyzed
for their ability to induce DNA synthesis of hepatocytes in primary
culture and to compete for binding to a soluble form of the huHGF
receptor.

10   A.   Protein quantification of wild-type huHGF and huHGF variants.

A specific two-site huHGF sandwich ELISA using two monoclonal
antibodies was used to quantify wild-type recombinant huHGF (WT
rhuHGF), single chain and protease substitution variants. Microtiter
plates (Maxisorb, Nunc) were coated with 10 mg/ml of a monoclonal
15   anti-rhuHGF antibody A 3.1.2 (IgG2a phenotype, affinity: 3.2×10^{-8}
mol) in 50 mM Carbonate buffer, pH 9.6, overnight at 4°C. After
blocking plates with 0.5 % BSA (Sigma), 0.01 % thimerosal in PBS, pH
7.4, and subsequent washes, duplicate serial dilutions of HGF samples
were prepared and in parallel a CHO-expressed rhuHGF (40-0.1 ng/mL)
20   was used as a standard. Fifty microliters of these dilutions were
simultaneously incubated with 50 mL of a 1:1500 diluted horseradish
peroxidase conjugated monoclonal anti-rhuHGF antibody B 4.3 (IgG1
phenotype, affinity: 1.3×10^{-8} mol) for 2 h at RT. The substrate was
prepared by adding 0.04 % o-phenylenediamine-dihydrochloride (Sigma)
25   and 0.012 % (v/v) hydrogen-peroxide (Sigma) to PBS and 100 ml were
added to the washed plates for 15 minutes at RT. The reaction was
stopped by adding 50 mL of 2.25 M sulfuric acid to each well. The
absorbance at 490 nm, with the absorbance at 405 nm subtracted as
background, was determined on a microtiter plate reader (Vmax,
30   Molecular Devices, Menlo Park, CA). The data was reduced using a
four-parameter curve-fitting program developed at Genentech, Inc.

An HGF polyclonal sandwich ELISA was used to quantify all kringle
deletion and C-terminal truncation variants. Briefly, microtiter
plates (Nunc) were coated with 5 mg/mL guinea pig polyclonal (anti
35   CHO-expressed rhuHGF) IgG antibody preparation (Genentech, Inc.) as
described above. This antibody recognizes rhuHGF as well as HGF
truncated forms when compared to visual inspection of Western blots,
making it ideal for monitoring HGF variants. Plates were blocked and

31

duplicate serial dilutions of 293 cell supernatants (1:103-6.106) were
added and incubated over night at 4°C. Purified CHO-expressed rhuHGF
(100-0.78 ng/mL) was used as a standard and incubated in parallel.
Plates were washed and incubated with a 1:500 dilution of the same

5   polyclonal antibody (approx. 400 ng/mL) but in this case horseradish
peroxidase conjugated for detection of the variants (see above).
Western blotting was performed to determine the size of the expressed
HGF variants. For this, SDS-polyacrylamide gel electrophoresis and
Western blotting were performed using standard methods with the

10 polyclonal IgG antibody preparation (500 ng/mL). A chemiluminescent
detection method (Amersham) and a goat anti-guinea pig IgG-horseradish
peroxidase conjugate (1:5000) were used for development of the blot as
described by the manufacturer.

    B.   Soluble HGF receptor binding assay.

15   Previous studies on HGF binding to hepatocytes have shown that
huHGF could bind to its cell surface receptor with high affinity
(Kd~24-32 pM; Higuchi and Nakamura, Biochem. Biophys. Res. Comm. 174,
831-838 (1991)). We preferred to examine HGF binding using a soluble
form of the receptor because of the nonspecific binding of HGF to cell

20 surface heparin sulfate proteoglycans [Naldini et al., EMBO J. 10,
2867-2878 (1991)].

    Cell supernatants (concentrated on Amicon filters if
concentration was below 600 ng/mL) were tested for their ability to
block in solution the binding of CHO-expressed ^{125}I rhuHGF (2-5 x 103

25 Ci/mmole, kindly provided by T. Zioncheck, Genentech, Inc.) to the
extracellular domain of the human HGF receptor (huHGFr) fused to the
Fc constant region of an human IgG, expressed and secreted from 293
cells.

    1.   Construction of huHGFr-IgG chimeras.

30   A full length cDNA clone encoding the huHGFr was constructed by
joining partial cDNAs isolated from cDNA libraries and from PCR
amplification. Coding sequences for amino acids 1-270 were isolated
from a human placental cDNA library (provided by T. Mason, Genentech)
screened with a 50 mer oligonucleotide (5'-

35 ATGAAGGCCCCGCTGTGCTTGCACCTGGCATCCTCGTGCTCCTGTTTACC-3') (SEQ. ID. NO:
16). Sequences encoding amino acids 809-1390 were isolated from a
human liver library (Stragagen) screened with the oligonucleotide
probe

(5'-CACTAGTTAGGATGGGGGACATGTCTGTCAGAGGATACTGCACTTGTCGGCATGAA CCGT-3').
(SEQ. ID. NO: 17)

     Conditions for plating libraries, and for hybridization and
washing filters were as described [Godowski et al., Proc. Natl. Acad.
Sci. USA 86, 8083-8087 (1989)]. PCR was used to isolate a cDNA clone
containing residues 271-808 of the HGFr (c-met) from A549 cells. Ten
μgs of total RNA was used for reverse transcription using a primer
specific to the HGFr (5'-TAGTACTAGCACTATGATGTCT -3') (SEQ. ID. NO: 18)
in a 100 μl reaction using Moloney murine leukemia virus reverse
transcriptase and buffers supplied by Bethesda Research Laboratories.
One-tenth of this reaction mixture was used for PCR amplification.
The PCR reaction was performed in a volume of 100 μl containing 10 μl
of the reverse transcriptase reaction, 10 mM KCl, 20 mM Tris-HCl (pH
8.8), 10 mM (NH4)SO4, 6 mM MgSO4, 0.1% Trition X-100, 1 U of Vent DNA
polymerase (New England Biolabs) and 50 pmol each of the forward
primer (5'-TTTACTTCTTGACGGTCCAAAG-3' (SEQ. ID. NO: 19) and the reverse
primer (5'-CAGGGGGAGTTGCAGATTCAGCTGT-3') (SEQ. ID. NO: 20). After
thirty cycles of denaturation (95°C, 1 min), annealing (55°C, 45 secs)
and extension (72°C, 2 min), the PCR product were recovered from low-
melting temperature agarose gels. The full-length HGFr cDNA was
subcloned into vector pRK7 (see WO 90/02798, published 22 March 1990)
and double-stranded DNA sequencing was performed by the
dideoxynucleotide method.

     The coding sequence of the extracellular domain of the huHGFr was
fused to those of the human IgG1 heavy chain in a two-step process.
PCR was used to generate a fragment with a unique BstEII site 3' to
the coding sequences of the HGFr amino acid 929. The 5' primer
(located in the vector upstream of the HGFr coding sequences) and the
3' primer (5'-AGTTTTGTCGGTGACCTGATCATTCTGATCTGGTTGAACTATTAC-3') (SEQ.
ID. NO: 21) were used in a 100 μl reaction as described above except
that the extension time at 72°C was 3 minutes, and 40 ng of the full
length HGFr expression vector was used as template. Following
amplification, the PCR product was joined to the human IgG-γ1 heavy
chain cDNA through a unique BstEII site in that construct [Bennett et
al., J. Biol. Chem. 266, 23060-23067 (1991)]. The resulting construct
contained the coding sequences of amino acids 1-929 of the huHGFr
fused via the BstEII site (adding the coding sequences for amino acids
V and T) to the coding sequences of amino acids 216-443 of the human

IgG-γ1 heavy chain. Sequencing of the construct was carried out as described above.

2. Binding assay.

The binding assay was performed in breakable microtiter plates (Nunc) coated o/n at 4°C with 1 mg/mL of rabbit-anti-human IgG Fc specific antibody (Jackson Immunoresearch) and plates were carefully washed with PBS containing 0.05% Tween 20 (Biorad). After blocking with PBS containing 0.1% BSA, in this same buffer, 50pM of 125I-rhuHGF in 25 mL per well were added. To each well 50 mL of serial dilutions (1:25-1:6000) of cell supernatants, purified CHO-expressed rhuHGF (25,000-0.064 pM) or medium were added in duplicates. Subsequently, 25 mL of 50 pM of HGF receptor:IgG fusion protein were added and the plates were incubated with gentle shaking. After 4 hours, when equilibrium was reached, plates were washed and wells were individually counted in a gamma-counter. The amount of nonspecifically bound radioactivity was estimated by incubating HGF receptor:IgG with a 500-fold excess of unlabelled rhuHGF. The dissociation constant (Kd) of each analogue was calculated at the IC50 from fitted inhibition curves essentially as described (DeBlasi et al., 1989 [?]) using the huHGF concentration determined by ELISA.

C. Biological assay.

The biological activity of WT huHGF and variants was measured by their abilities to induce DNA synthesis of rat hepatocytes in primary culture. Hepatocytes were isolated according to published perfusion techniques with minor modifications [Garrison and Haynes, *J. Biol. Chem.* 150, 2269-277 (1975)]. Briefly, the livers of female Sprague Dawley rats (160-180g) were perfused through the portal vein with 100 mL of Ca^{++} free Hepes buffered saline containing 0.02% Collagenase type IV (Sigma). After 20 minutes the liver was removed, placed in buffer, gently stirred to separate hepatocytes from connective tissue and blood vessels, and filtered through nylon mesh. Cells were then washed by centrifugation, resuspended at 1×10^5 cells/mL in Williams Media E (Gibco) containing Penicillin (100 U/ml), Streptomycin (100 mg/mL), L-Glutamine (2mM), trace elements (0.01%), transferrin (10 mg/mL) and Aprotinin (1 mg/mL). Hepatocytes were incubated in 96-well microtiter plates (Falcon) in the presence of duplicate serial dilutions of either purified CHO-expressed rhuHGF (1-0.031 mg/mL), 293 supernatants (1:4-1:256) or medium. After 48 hours incubation at

37°C, 0.5 mCi 3H-TdR (15 Ci/mmole, Amersham) was added to each well
and incubated for an additional 16 hours. Cells were harvested on
filter papers, which were washed, dried and counted in a Beckman
counter after addition of scintillation liquid. For each huHGF

5 variant, the specific activity (SA) expressed in units/mg was
calculated at half-maximal proliferation (defined as 1 unit/mL) using
the HGF concentration obtained in ELISA.

D.   Induction of tyrosine phosphorylations on A549 cells.

Human lung carcinoma cells (A549) monolayers were cultured in
10 RPMI 1640 medium containing 10% fetal bovine serum and maintained at
37°C in a humidified atmosphere with 5% CO_2. Serum-starved cells were
incubated without or with 200 ng/mL rhuHGF for 5 minutes at 37°C and
extracted with lysis buffer containing 50 mM Hepes, 150 mM NaCl, 1.5
mM $MgCl_2$, 1 mM EGTA, 10 % Glycerol, 1 % Triton X-100 and a cocktail

15 of protease inhibitors. The lysates were immunoprecipitated with
anti-Met COOH antibodies and blotted with anti-phosphotyrosine
antibodies (see Western blotting above).

EXAMPLE 3

20 Analysis of Cleavage Site Mutants

The cleavage site of proteases commonly contains a basic residue
at position P1 and two hydrophobic amino acid resides in positions P'1
and P'2, which follow the cleaved peptide bond. The proposed cleavage
site of huHGF (P1 R494, P'1 V495, P'2 V496) fits this consensus. We

25 chose to try to block cleavage of huHGF by replacing the P1 R494 with
either D, E, or A. The major form of WT rhuHGF expressed in these
cells is cleaved into two-chain material as judged by the presence of
the α-chain with an apparent molecular mass of 69 kDa (Fig. 2). Each
of these mutations appeared to block processing of rhuHGF because

30 under reducing conditions these variants migrated as a single band at
94 kDa, the predicted size of single-chain HGF. These variants
totally lacked the ability to induce the proliferation of hepatocytes
in primary culture (Fig. 3A). However, when these variants were
analyzed for their ability to compete with WT rhuHGF for binding to

35 the HGF receptor:IgG fusion protein, their inhibition curves were
roughly similar to that of WT rhuHGF (Fig. 3B). The Kd determined
from these curves showed that WT rhuHGF binds to the fusion protein
with high affinity (50-70pM) whereas all single chain variants showed

35

approximately a 2- to 10-fold higher Kd (100-500pM) compared to WT
rhuHGF. Results from at least three independent assays are summarized
in Table I as residual hepatocyte proliferative activity and receptor
binding capacity compared to WT rhuHGF.

5   Our binding studies showed that WT rhuHGF bound to the soluble
receptor fusion protein with a single class of high affinity binding
sites (50-70 pM), similar to those found on hepatocytes by Higushi and
Nakamura (1991). However, binding of HGF on cells may slightly be
different since the soluble receptor is actually a dimer held together
10   by the disulfide bridge of the hinge in the Fc portion of the IgGA.

Direct comparison of specific activity (SA) versus Kd ratios of
all single chain variants showed they were inactive at the highest
concentration tested (SA< 3%) while receptor binding affinities were
only decreased by a factor of 2-3.

15   These results argue strongly that cleavage of HGF into the two-
chain form is required for mitogenic activity, i.e. that single-chain
HGF is a promitogen and that the uncleaved form of HGF binds to the
HGF receptor, albeit with a reduced affinity.

The major form of HGF isolated from placenta [Hernandez et al.,
20   (1992) J. Cell Physiol., in press] or expressed in transfected COS
cells [Rubin et al., Proc. Natl. Acad. Sci. USA 88, 415-419 (1991)] is
in single-chain form. When tested in mitogenic assays, this single-
chain form of HGF is found to be biologically active. Taken together
with our data, this suggests that this single-chain HGF is activated
25   to the two-chain form during the mitogenic assay.

A second observation is that single-chain variants retain
substantial capacity to bind to the HGF receptor, as suggested by our
competition binding assays. This raises the interesting possibility
that single-chain HGF may be bound to cell-surface HGF receptor in
30   vivo in an inactive state and can subsequently be cleaved to the
active double-chain form by the appropriate protease.

EXAMPLE 4
The Effects of Protease Domain Mutations

35   To elucidate the functional importance of the protease domain of
HGF, several single, double and triple mutations were made in order to
reconstitute a potential serine-protease active site. The
construction of these variants is described in Example 1.

We replaced HGF residues Q534 with H, Y673 with S, or V692 with S
as either single, double or triple mutations. The analysis of their
effects on mitogenic activity and receptor binding showed that the
single mutation Q534H did not significantly alter either SA (5.2 x 104

5   Units/mg) or Kd (60 pM) when compared to wt rhuHGF (respectively 3.3
104 Units/mg and 70 pM) whereas Y673S and V692S exhibited SA reduced
approximately 5- and 10-fold, respectively. In fact, these two
variants never reached the maximum plateau seen with WT rhuHGF
(approximately 50 % of wt rhuHGF plateau). Interestingly, these

10   variants showed a Kd similar to WT rhuHGF. All other double and
triple variants also retained the ability to bind the HGF receptor but
they clearly showed a reduced SA (Table I). The residual SA of the
double variants Q534H,Y673S and Y673S,V692S and of the triple variant
Q534H,Y673S,V692S were less than 3 % compared to WT rhuHGF. However,

15   the Kd of these variants was not significantly different from WT
rhuHGF (Table I). These variants indicate that mutations within the
β-chain of HGF block mitogenic activity but they are still able to
bind to the HGF receptor. Thus, it appears that these mutants are
defective in an activity subsequent to receptor binding.

20   These results show that although the β-chain is not required for
receptor binding, certain residues (e.g. Y673 and V692) are critical
for the structure and/or activity of HGF. Substitution of the
nonpolar residue V692 with the polar residue S might have caused a
structural transition if new hydrogen bonds to the active site residue

25   D594, as found in serine-proteases, have been introduced.
Substitution of Y673 with the smaller residue S might also introduce
some local structural modifications. On the other hand, replacement
of the polar residue Q534 by another polar residue H of similar size
would not likely cause a drastic difference in the HGF conformation as

30   this residue should be exposed; indeed the Q534H variant was similar
to rhuHGF (Table I).

EXAMPLE 5

The Effect of C-terminal and Kringle Deletions

35   In order to ascertain whether the α-chain is required for HGF
binding or activity, C-terminal truncations were made as described in
Example 1, resulting in variants containing either the α-chain alone,

37

or variants truncated after the third (N-384) or second (N-303) Kringles.

A number of C-terminal truncations of HGF were made by deleting either the β-chain or the β-chain in addition to a progressive number of kringles as depicted in Fig. 1. One variant (N-207) corresponding to the N-terminal domain with the first Kringle did not express the protein to levels detectable either by Western blotting or ELISA using a polyclonal antibody preparation and thus was not investigated further. Expression of the variants containing the first two Kringles (N-303), three Kringles (N-384) or the complete α-chain of HGF was as low as 250-600 ng/mL. A summary of the residual SA and Kd compared to WT rhuHGF of these variants is presented in Table I. At the concentration tested no activity above background levels was observed indicating that these variants lost their biological activity.

However, binding competition showed that variants N-303, N-384 or the α-chain still retained substantial binding capacity (up to 23 % compared to WT rhuHGF binding). Thus, the N-terminal 272 residues of HGF (the mature form of variant N-303) are sufficient for high affinity binding to the HGF receptor.

Results from deleting each kringle domain are shown in Table 1. Deletion of the first Kringle (variant ΔK1) of HGF affected biological activity most, showing at least a 100-fold reduction (SA< 0.2% of wt rhuHGF). Similarly, binding of this variant was also affected as it failed to compete for binding with wt rhuHGF up to 2 mg/mL. Deletion of all other Kringles (variants ΔK2, ΔK3 or ΔK4) also induces severely reduced mitogenic activity (Table I). However, the Kds of these deletion variants remained close to that observed with wt rhuHGF.

These data show that Kringles K3 and K4 are not required for receptor binding. Our data support the previous observations by Miyazawa et al., 1991 supra and Chan et al., 1991 supra, in the sense that variant N-303, which in amino acid sequence is very similar to HGF/NK2, retains the ability to compete efficiently for binding to the HGF receptor (Kd~280 pM). Furthermore, the observations that N-303 is sufficient to bind to the receptor and that the second Kringle is not required for binding the HGF receptor (in the context of the remainder of the molecule) suggest that the receptor binding domain is contained within the finger and first Kringle of huHGF.

Unfortunately, we have not been able to detect expression of this

variant using our polyclonal antisera suggesting that variant N-207
(deletion after the first kringle) was not expressed in 293 cells.

EXAMPLE 6

5   ### Induction of Tyrosine-Phosphorylation of the huHGF Receptor

We determined if variants R494E or Y673S,V692S, which bind the
HGF receptor _in vitro_ but are defective for mitogenic activity, could
stimulate tyrosine-phosphorylation of the HGF receptor in A549 cells.
Serum starved cells were treated with purified WT rhuHGF or variants
10   and immunoprecipitates of the HGF receptor were blotted and probed
with phosphotyrosine antibodies. Stimulation with wt rhuHGF led to the
phosphorylation on tyrosine of the 145 kDa β-subunit of the HGF
receptor (Fig. 4). Both variants exhibited a reduced ability to
induce phosphorylation of the HGF receptor.

15   Stimulation of tyrosine phosphorylation on the HGF receptor β-
subunit by HGF was previously reported [Bottaro _et al._, _Science_ 251,
802-804 (1991), Naldini _et al._, 1991 _supra_]. The present data show
that variants R494E and Y673S,V692S can bind the soluble HGF receptor:
IgG protein in vitro but are not efficient in stimulating tyrosine-
20   phosphorylation in A549 cells. One interpretation of this result is
that these variants are capable of binding the HGF receptor on A549
cells, but are defective in a function required to induce efficient
phosphorylation, e.g. receptor dimerization. It has been shown for
other receptor proteins with an intrinsic tyrosine kinase such as the
25   epithelial and platelet-derived growth factor that receptor-receptor
interactions or dimerization is required for activation of kinase
function [see for review Ulrich and Schlessinger, _Cell_ 61 203-212
(1990)]. Alternatively, these variants may not be able to bind the
cell-surface associated HGF receptor.

30   The unique structure of HGF suggests that there may be multiple
events that regulate the biological activity of this molecule. An
early stage of regulation may be the cleavage step to generate the
biologically active two-chain form. Interestingly, cleavage may not
simply regulate receptor binding but rather control a subsequent event
.35   required for activating the HGF receptor. Our data also suggest that
the β-chain, while not absolutely required for receptor binding
contributes to a receptor activation step. These variants may be
useful in dissecting the signalling events at the HGF receptor.

EXAMPLE 7

Hairpin Domain and Kringle 1 Domain Variants

The huHGF variants listed in Tables 2 and 3 were generated, and
their specific activities (SA) and Kd ratios were determined
5   essentially as described in the foregoing examples.

- - - - - - - - - - - -

Although the foregoing refers to particular preferred
embodiments, it will be understood that the present invention is not
so limited. It will occur to those ordinarily skilled in the art that
10   various modifications may be made to the disclosed embodiments without
diverting from the overall concept of the invention. All such
modifications are intended to be within the scope of the present
invention.

15

Table 1

Variants (var)	SA var/SA wt +/- S.D.	Kdwt/Kdvar +/- S.D.
Single-chain		
R494A	<0.03	0.32 +/- 0.18
R494D	<0.03	0.51 +/- 0.21
R494E	<0.02	0.31 +/- 0.13
Protease		
Q534H	1.19 +/- 0.44	1.48 +/- 0.85
Y673S	0.27 +/- 0.07*	1.35 +/- 0.72
V692S	0.08 +/- 0.04	1.02 +/- 0.13
Q534H,Y673S	<0.03	2.24 +/- 1.11
Y673S,V692S	<0.02	1.76 +/- 0.63
Q534H, Y673S, V692S	<0.02	1.91 +/- 1.28
C-terminal truncation		
N-303	<0.05	0.23 +/- 0.03
N-384	<0.05	0.25 +/- 0.02
α-chain	<0.04	0.25 +/- 0.03
Kringle deletion		
ΔK1	<0.002	<0.03
ΔK2	<0.05	0.41 +/- 0.18
ΔK3	<0.03	0.56 +/- 0.36
ΔK4	<0.07	0.86 +/- 0.46

* means that the mitogenic activity of the variant did not reach the same absolute level as wild-type huHGF.

Table 2

HGF variant	n	SA mut/wt +/-SD	n	Kdwt/mut
wt rhuHGF	3	1	3	1
K137A	3	1.12 +/- 0.10	3	0.98 +/- 0.04
K144A,K148A	3	0.93 +/- 0.16	3	1.03 +/- 0.08
E159A	3	< 0.02	3	< 0.03
S161A	3	0.15 +/- 0.03*	3	0.06 +/- 0.04
F162A,L163A,S165A,S166A	3	< 0.02	3	0.05 +/- 0.02
F162A	3	0.04 +/1 0.01*	3	0.05 +/- 0.01
L163A,S165A,S166A	3	0.14 +/- 0.08	3	1.10 +/- 0.04
Y167A	3	1.22 +/- 0.22*	3	0.92 +/- 0.06
Y167F	3	0.38 +/- 0.03*	3	1.02 +/- 0.02
delta5-Y167A	3	< 0.02	3	< 0.02
delta5-Y167F	3	0.11 +/- 0.02	3	1.01 +/- 0.13
R168A	3	0.83 +/- 0.07	3	0.98 +/- 0.04
Q173A,E174A,N175A	3	0.33 +/- 0.07*	3	0.21 +/- 0.03
Q173A	3	0.13 +/- 0.03*	3	0.15 +/- 0.05
E174A	3	0.84 +/- 0.11	3	0.99 +/- 0.02
R181A,E183A,E184A	3	0.92 +/- 0.08	3	0.95 +/- 0.04
N193A,E195A,R197A	3	< 0.02	3	< 0.04
N193A	3	0.45 +/- 0.12	3	0.62 +/- 0.20
R195A	3	0.10 +/- 0.06	3	0.17 +/- 0.05
R197A	3	< 0.01	3	< 0.03
Y198A	3	0.88 +/- 0.06	2	0.77

* means that the mitogenic activity of the variant did not reach the same absolute level as wild-type huHGF.

42

Table 3

Variants with alterations near or within the Hairpin Domain

HGF variant	n	SA +/- mut/wt	n	Kd wt/mut
wt rhuHGF	3	1	3	1
delta-hairpin	3	< 0.01	3	< 0.02
K34A,R35A,R36A	3	0.71 +/- 0.33	3	0.95 +/- 0.03
K52A,D54A	3	0.32 +/- 0.03*	3	0.95 +/- 0.03
K52A	3	0.19 +/- 0.03	3	0.89 +/- 0.05
D54A	3	0.13 +/- 0.01	3	0.79 +/- 0.10
K58A,K60A,K62A,K63A,N65A	3	0.96 +/- 0.12	3	0.99 +/- 0.07
N72A,R76A,N77A,K78A	3	0.04 +/- 0.07	3	0.83 +/- 0.11
K109A,K110A,E111A	3	0.98 +/- 0.04	3	0.78 +/- 0.02
H114A,E115A,D117A	3	< 0.02	3	< 0.04
H114A	3	0.16 +/- 0.04*	2	0.59
E115A	3	0.20 +/- 0.02*	2	0.47
D117A	3	0.02*	2	0.14
R126A,N127A	3	0.33 +/- 0.14	3	0.87 +/- 0.12

* means that the mitogenic activity of the variant did not reach the same absolute level as wild-type huHGF.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5   (i) APPLICANT: Genentech, Inc., Godowski, Paul J., Lokker, Natalie
A., Mark, Melanie R.

        (ii) TITLE OF INVENTION: HEPATOCYTE GROWTH FACTOR VARIANTS

10   (iii) NUMBER OF SEQUENCES: 21

        (iv) CORRESPONDENCE ADDRESS:
            (A) ADDRESSEE: Genentech, Inc.
            (B) STREET: 460 Point San Bruno Blvd
15   (C) CITY: South San Francisco
            (D) STATE: California
            (E) COUNTRY: USA
            (F) ZIP: 94080

20   (v) COMPUTER READABLE FORM:
            (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
            (B) COMPUTER: IBM PC compatible
            (C) OPERATING SYSTEM: PC-DOS/MS-DOS
            (D) SOFTWARE: patin (Genentech)
25
        (vi) CURRENT APPLICATION DATA:
            (A) APPLICATION NUMBER:
            (B) FILING DATE:
            (C) CLASSIFICATION:
30
        (vii) PRIOR APPLICATION DATA:
            (A) APPLICATION NUMBER: 07/884811
            (B) FILING DATE: 18-MAY-92

35   (vii) PRIOR APPLICATION DATA:
            (A) APPLICATION NUMBER: 07/885971
            (B) FILING DATE: 18-MAY-92

        (viii) ATTORNEY/AGENT INFORMATION:
40   (A) NAME: Dreger, Ginger R.
            (B) REGISTRATION NUMBER: 33,055
            (C) REFERENCE/DOCKET NUMBER: 755,779P1

        (ix) TELECOMMUNICATION INFORMATION:
45   (A) TELEPHONE: 415/225-3216
            (B) TELEFAX: 415/952-9881
            (C) TELEX: 910/371-7168

        (2) INFORMATION FOR SEQ ID NO:1:
50
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 4732 bases
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
55   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT 50

TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC 100

TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG 150

ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA 200

TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC 250

ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT 300

AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC 350

TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC 400

GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA 450

TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA 500

AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC 550

AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT 600

TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT 650

CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA 700

TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA 750

GTCTATAGGC CCACCCCCTT GGCTTCGTTA GAACGCGGCT ACAATTAATA 800

CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA 850

CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC 900
```

ACCTCGGTTC TATCGATTGA ATTCCCCGGG GATCCTCTAG AGTCGACCTG 950

CAGAAGCTTG CCTCGAGGCA AGCTTGGCCG CCATGGCCCA ACTTGTTTAT 1000

TGCAGCTTAT AATGGTTACA AATAAAGCAA TAGCATCACA AATTTCACAA 1050

ATAAAGCATT TTTTTCACTG CATTCTAGTT GTGGTTTGTC CAAACTCATC 1100

AATGTATCTT ATCATGTCTG GATCGATCGG GAATTAATTC GGCGCAGCAC 1150

CATGGCCTGA AATAACCTCT GAAAGAGGAA CTTGGTTAGG TACCTTCTGA 1200

GGCGGAAAGA ACCAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC 1250

CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT 1300

CAGCAACCAG GTGTGGAAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG 1350

CAAAGCATGC ATCTCAATTA GTCAGCAACC ATAGTCCCGC CCCTAACTCC 1400

GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG 1450

GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT 1500

GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG 1550

CAAAAAGCTG TTAACAGCTT GGCACTGGCC GTCGTTTTAC AACGTCGTGA 1600

CTGGGAAAAC CCTGGCGTTA CCCAACTTAA TCGCCTTGCA GCACATCCCC 1650

CCTTCGCCAG CTGGCGTAAT AGCGAAGAGG CCCGCACCGA TCGCCCTTCC 1700

CAACAGTTGC GTAGCCTGAA TGGCGAATGG CGCCTGATGC GGTATTTTCT 1750

CCTTACGCAT CTGTGCGGTA TTTCACACCG CATACGTCAA AGCAACCATA 1800

GTACGCGCCC TGTAGCGGCG CATTAAGCGC GGCGGGTGTG GTGGTTACGC 1850

```
GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC TCCTTTCGCT 1900

TTCTTCCCTT CCTTTCTCGC CACGTTCGCC GGCTTTCCCC GTCAAGCTCT 1950

AAATCGGGGG CTCCCTTTAG GGTTCCGATT TAGTGCTTTA CGGCACCTCG 2000

ACCCCAAAAA ACTTGATTTG GGTGATGGTT CACGTAGTGG GCCATCGCCC 2050

TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT TCTTTAATAG 2100

TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC TCGGGCTATT 2150

CTTTTGATTT ATAAGGGATT TTGCCGATTT CGGCCTATTG GTTAAAAAAT 2200

GAGCTGATTT AACAAAAATT TAACGCGAAT TTTAACAAAA TATTAACGTT 2250

TACAATTTTA TGGTGCACTC TCAGTACAAT CTGCTCTGAT GCCGCATAGT 2300

TAAGCCAACT CCGCTATCGC TACGTGACTG GGTCATGGCT GCGCCCCGAC 2350

ACCCGCCAAC ACCCGCTGAC GCGCCCTGAC GGGCTTGTCT GCTCCCGGCA 2400

TCCGCTTACA GACAAGCTGT GACCGTCTCC GGGAGCTGCA TGTGTCAGAG 2450

GTTTTCACCG TCATCACCGA AACGCGCGAG GCAGTATTCT TGAAGACGAA 2500

AGGGCCTCGT GATACGCCTA TTTTTATAGG TTAATGTCAT GATAATAATG 2550

GTTTCTTAGA CGTCAGGTGG CACTTTTCGG GGAAATGTGC GCGGAACCCC 2600

TATTTGTTTA TTTTTCTAAA TACATTCAAA TATGTATCCG CTCATGAGAC 2650

AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA GAGTATGAGT 2700

ATTCAACATT TCCGTGTCGC CCTTATTCCC TTTTTTGCGG CATTTTGCCT 2750

TCCTGTTTTT GCTCACCCAG AAACGCTGGT GAAAGTAAAA GATGCTGAAG 2800
```

47

```
ATCAGTTGGG TGCACGAGTG GGTTACATCG AACTGGATCT CAACAGCGGT 2850

AAGATCCTTG AGAGTTTTCG CCCCGAAGAA CGTTTTCCAA TGATGAGCAC 2900

TTTTAAAGTT CTGCTATGTG GCGCGGTATT ATCCCGTGAT GACGCCGGGC 2950

AAGAGCAACT CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTGAG 3000

TACTCACCAG TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA 3050

ATTATGCAGT GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC 3100

TTCTGACAAC GATCGGAGGA CCGAAGGAGC TAACCGCTTT TTTGCACAAC 3150

ATGGGGGATC ATGTAACTCG CCTTGATCGT TGGGAACCGG AGCTGAATGA 3200

AGCCATACCA AACGACGAGC GTGACACCAC GATGCCAGCA GCAATGGCAA 3250

CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCGG 3300

CAACAATTAA TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT 3350

GCGCTCGGCC CTTCCGGCTG GCTGGTTTAT TGCTGATAAA TCTGGAGCCG 3400

GTGAGCGTGG GTCTCGCGGT ATCATTGCAG CACTGGGGCC AGATGGTAAG 3450

CCCTCCCGTA TCGTAGTTAT CTACACGACG GGGAGTCAGG CAACTATGGA 3500

TGAACGAAAT AGACAGATCG CTGAGATAGG TGCCTCACTG ATTAAGCATT 3550

GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA 3600

CTTCATTTTT AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT 3650

CATGACCAAA ATCCCTTAAC GTGAGTTTTC GTTCCACTGA GCGTCAGACC 3700

CCGTAGAAAA GATCAAAGGA TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA 3750
```

48

```
          ATCTGCTGCT TGCAAACAAA AAAACCACCG CTACCAGCGG TGGTTTGTTT 3800

          GCCGGATCAA GAGCTACCAA CTCTTTTTCC GAAGGTAACT GGCTTCAGCA 3850
     5
          GAGCGCAGAT ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC 3900

          CACTTCAAGA ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT 3950
    10
          GTTACCAGTG GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG 4000
    15
          ACTCAAGACG ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG 4050

          GGTTCGTGCA CACAGCCCAG CTTGGAGCGA ACGACCTACA CCGAACTGAG 4100
    20
          ATACCTACAG CGTGAGCATT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA 4150

          AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG 4200
    25
          AGGGAGCTTC CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT 4250
    30
          TCGCCACCTC TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC 4300

          GGAGCCTATG GAAAAACGCC AGCAACGCGG CCTTTTTACG GTTCCTGGCC 4350
    35
          TTTTGCTGGC CTTTTGCTCA CATGTTCTTT CCTGCGTTAT CCCCTGATTC 4400

          TGTGGATAAC CGTATTACCG CCTTTGAGTG AGCTGATACC GCTCGCCGCA 4450
    40
          GCCGAACGAC CGAGCGCAGC GAGTCAGTGA GCGAGGAAGC GGAAGAGCGC 4500
    45
          CCAATACGCA AACCGCCTCT CCCCGCGCGT TGGCCGATTC ATTAATCCAG 4550

          CTGGCACGAC AGGTTTCCCG ACTGGAAAGC GGGCAGTGAG CGCAACGCAA 4600
    50
          TTAATGTGAG TTACCTCACT CATTAGGCAC CCCAGGCTTT ACACTTTATG 4650

          CTTCCGGCTC GTATGTTGTG TGGAATTGTG AGCGGATAAC AATTTCACAC 4700
    55
```

49

AGGAAACAGC TATGACCATG ATTACGAATT AA 4732

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 47 bases
            (B) TYPE: nucleic acid
10   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15

        TTGGAATCCC ATTTACAACC TCGAGTTGTT TCGTTTTGGC ACAAGAT 47

20   (2) INFORMATION FOR SEQ ID NO:3:

         (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 bases
            (B) TYPE: nucleic acid
25   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear.

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

30

        GAATCCCATT TACGACGTCC AATTGTTTCG 30

35   (2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 26 bases
            (B) TYPE: nucleic acid
40   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

45

        CCCATTTACA ACTGCCAATT GTTTCG 26

50   (2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 bases
            (B) TYPE: nucleic acid
55   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

   AGAAGGGAAA CAGTGTCGTG CA 22
5

      (2) INFORMATION FOR SEQ ID NO:6:

10   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 bases
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
15
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

   AGTGGGCCAC CAGAATCCCC CT 22
20

      (2) INFORMATION FOR SEQ ID NO:7:

25   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 23 bases
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
30
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

   TCCACGACCA GGAGAAATGA CAC 23
35

      (2) INFORMATION FOR SEQ ID NO:8:

40   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 bases
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
45
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

   GCATTCAACT TCTGAGTTTC TAATGTAGTC 30
50

      (2) INFORMATION FOR SEQ ID NO:9:

55   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 bases

                        (B) TYPE: nucleic acid
                        (C) STRANDEDNESS: single
                        (D) TOPOLOGY: linear

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

           CATAGTATTG TCAGCTTCAA CTTCTGAACA 30

10

           (2) INFORMATION FOR SEQ ID NO:10:

                (i) SEQUENCE CHARACTERISTICS:
15   (A) LENGTH: 30 bases
                     (B) TYPE: nucleic acid
                     (C) STRANDEDNESS: single
                     (D) TOPOLOGY: linear

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

           TCCATGTGAC ATATCTTCAG TTGTTTCCAA 30

25

           (2) INFORMATION FOR SEQ ID NO:11:

                (i) SEQUENCE CHARACTERISTICS:
30   (A) LENGTH: 30 bases
                     (B) TYPE: nucleic acid
                     (C) STRANDEDNESS: single
                     (D) TOPOLOGY: linear

35   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

           TGTGGTATCA CCTTCATCTT GTCCATGTGA 30

40

           (2) INFORMATION FOR SEQ ID NO:12:

                (i) SEQUENCE CHARACTERISTICS:
45   (A) LENGTH: 24 bases
                     (B) TYPE: nucleic acid
                     (C) STRANDEDNESS: single
                     (D) TOPOLOGY: linear

50   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

           ACCTTGGATG CATTAAGTTG TTTC 24

55

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 bases
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTGTCCATGT GATTAATCAC AGT 23

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 bases
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTCGTGTTG GGATCCCATT TACCTATCGC AATTG 35

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10596 bases
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT 50

TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC 100

TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG 150

ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA 200

TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC 250

```
ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT 300

AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC 350

TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC 400

GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA 450

TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA 500

AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC 550

AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT 600

TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT 650

CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA 700

TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA 750

GTCTATAGGC CCACCCCCTT GGCTTCGTTA GAACGCGGCT ACAATTAATA 800

CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA 850

CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC 900

ACCTCGGTTC TATCGATTCT CGAGAATTAA TTCAAGCTTG CGGCCGCAGC 950

TTGGCCGCCA TGGCCCAACT TGTTTATTGC AGCTTATAAT GGTTACAAAT 1000

AAAGCAATAG CATCACAAAT TTCACAAATA AAGCATTTTT TTCACTGCAT 1050

TCTAGTTGTG GTTTGTCCAA ACTCATCAAT GTATCTTATC ATGTCTGGAT 1100

CGATCGGGAA TTAATTCGGC GCAGCACCAT GGCCTGAAAT AACCTCTGAA 1150

AGAGGAACTT GGTTAGGTAC CTTCTGAGGC GGAAAGAACC AGCTGTGGAA 1200
```

54

```
       TGTGTGTCAG TTAGGGTGTG GAAAGTCCCC AGGCTCCCCA GCAGGCAGAA 1250

       GTATGCAAAG CATGCATCTC AATTAGTCAG CAACCAGGTG TGGAAAGTCC 1300
   5
       CCAGGCTCCC CAGCAGGCAG AAGTATGCAA AGCATGCATC TCAATTAGTC 1350

  10   AGCAACCATA GTCCCGCCCC TAACTCCGCC CATCCCGCCC CTAACTCCGC 1400

       CCAGTTCCGC CCATTCTCCG CCCCATGGCT GACTAATTTT TTTTATTTAT 1450
  15
       GCAGAGGCCG AGGCCGCCTC GGCCTCTGAG CTATTCCAGA AGTAGTGAGG 1500

       AGGCTTTTTT GGAGGCCTAG GCTTTTGCAA AAAGCTGTTC ACGTGATGAA 1550
  20
       TTCTCATGTT TGACAGCTTA TCATCGATAG ATCCTCACAG GCCGCACCCA 1600

  25   GCTTTTCTTC CGTTGCCCCA GTAGCATCTC TGTCTGGTGA CCTTGAAGAG 1650

       GAAGAGGAGG GGTCCCGAGA ATCCCCATCC CTACCGTCCA GCAAAAAGGG 1700
  30
       GGACGAGGAA TTTGAGGCCT GGCTTGAGGC TCAGGACGCA AATCTTGAGG 1750

  35   ATGTTCAGCG GGAGTTTTCC GGGCTGCGAG TAATTGGTGA TGAGGACGAG 1800

       GATGGTTCGG AGGATGGGGA ATTTTCAGAC CTGGATCTGT CTGACAGCGA 1850

  40   CCATGAAGGG GATGAGGGTG GGGGGGCTGT TGGAGGGGGC AGGAGTCTGC 1900

       ACTCCCTGTA TTCACTGAGC GTCGTCTAAT AAAGATGTCT ATTGATCTCT 1950
  45
       TTTAGTGTGA ATCATGTCTG ACGAGGGGCC AGGTACAGGA CCTGGAAATG 2000

       GCCTAGGAGA GAAGGGAGAC ACATCTGGAC CAGAAGGCTC CGGCGGCAGT 2050
  50
       GGACCTCAAA GAAGAGGGGG TGATAACCAT GGACGAGGAC GGGGAAGAGG 2100

  55   ACGAGGACGA GGAGGCGGAA GACCAGGAGC CCCGGGCGGC TCAGGATCAG 2150
```

55

```
      GGCCAAGACA TAGAGATGGT GTCCGGAGAC CCCAAAAACG TCCAAGTTGC 2200

      ATTGGCTGCA AAGGGACCCA CGGTGGAACA GGAGCAGGAG CAGGAGCGGG 2250

      AGGGGCAGGA GCAGGAGGGG CAGGAGCAGG AGGAGGGGCA GGAGCAGGAG 2300

      GAGGGGCAGG AGGGGCAGGA GGGGCAGGAG GGGCAGGAGC AGGAGGAGGG 2350

      GCAGGAGCAG GAGGAGGGGC AGGAGGGGCA GGAGGGGCAG GAGCAGGAGG 2400

      AGGGGCAGGA GCAGGAGGAG GGGCAGGAGG GGCAGGAGCA GGAGGAGGGG 2450

      CAGGAGGGGC AGGAGGGGCA GGAGCAGGAG GAGGGGCAGG AGCAGGAGGA 2500

      GGGGCAGGAG GGGCAGGAGC AGGAGGAGGG GCAGGAGGGG CAGGAGGGGC 2550

      AGGAGCAGGA GGAGGGGCAG GAGCAGGAGG GGCAGGAGGG GCAGGAGGGG 2600

      CAGGAGCAGG AGGGGCAGGA GCAGGAGGAG GGGCAGGAGG GGCAGGAGGG 2650

      GCAGGAGCAG GAGGGGCAGG AGCAGGAGGG GCAGGAGCAG GAGGGGCAGG 2700

      AGCAGGAGGG GCAGGAGGGG CAGGAGCAGG AGGGGCAGGA GGGGCAGGAG 2750

      CAGGAGGGGC AGGAGGGGCA GGAGCAGGAG GAGGGGCAGG AGGGGCAGGA 2800

      GCAGGAGGAG GGGCAGGAGG GGCAGGAGCA GGAGGGGCAG GAGGGGCAGG 2850

      AGCAGGAGGG GCAGGAGGGG CAGGAGCAGG AGGGGCAGGA GGGGCAGGAG 2900

      CAGGAGGAGG GGCAGGAGCA GGAGGGGCAG GAGCAGGAGG TGGAGGCCGG 2950

      GGTCGAGGAG GCAGTGGAGG CCGGGGTCGA GGAGGTAGTG GAGGCCGGGG 3000

      TCGAGGAGGT AGTGGAGGCC GCCGGGGTAG AGGACGTGAA AGAGCCAGGG 3050

      GGGGAAGTCG TGAAAGAGCC AGGGGGAGAG GTCGTGGACG TGGAGAAAAG 3100
```

```
AGGCCCAGGA GTCCCAGTAG TCAGTCATCA TCATCCGGGT CTCCACCGCG 3150

CAGGCCCCCT CCAGGTAGAA GGCCATTTTT CCACCCTGTA GGGGAAGCCG 3200

ATTATTTTGA ATACCACCAA GAAGGTGGCC CAGATGGTGA GCCTGACGTG 3250

CCCCCGGGAG CGATAGAGCA GGGCCCCGCA GATGACCCAG GAGAAGGCCC 3300

AAGCACTGGA CCCCGGGGTC AGGGTGATGG AGGCAGGCGC AAAAAAGGAG 3350

GGTGGTTTGG AAAGCATCGT GGTCAAGGAG GTTCCAACCC GAAATTTGAG 3400

AACATTGCAG AAGGTTTAAG AGCTCTCCTG GCTAGGAGTC ACGTAGAAAG 3450

GACTACCGAC GAAGGAACTT GGGTCGCCGG TGTGTTCGTA TATGGAGGTA 3500

GTAAGACCTC CCTTTACAAC CTAAGGCGAG GAACTGCCCT TGCTATTCCA 3550

CAATGTCGTC TTACACCATT GAGTCGTCTC CCCTTTGGAA TGGCCCCTGG 3600

ACCCGGCCCA CAACCTGGCC CGCTAAGGGA GTCCATTGTC TGTTATTTCA 3650

TGGTCTTTTT ACAAACTCAT ATATTTGCTG AGGTTTTGAA GGATGCGATT 3700

AAGGACCTTG TTATGACAAA GCCCGCTCCT ACCTGCAATA TCAGGGTGAC 3750

TGTGTGCAGC TTTGACGATG GAGTAGATTT GCCTCCCTGG TTTCCACCTA 3800

TGGTGGAAGG GGCTGCCGCG GAGGGTGATG ACGGAGATGA CGGAGATGAA 3850

GGAGGTGATG GAGATGAGGG TGAGGAAGGG CAGGAGTGAT GTAACTTGTT 3900

AGGAGACGCC CTCAATCGTA TTAAAAGCCG TGTATTCCCC CGCACTAAAG 3950

AATAAATCCC CAGTAGACAT CATGCGTGCT GTTGGTGTAT TTCTGGCCAT 4000

CTGTCTTGTC ACCATTTTCG TCCTCCCAAC ATGGGGCAAT TGGGCATACC 4050
```

CATGTTGTCA CGTCACTCAG CTCCGCGCTC AACACCTTCT CGCGTTGGAA 4100

AACATTAGCG ACATTTACCT GGTGAGCAAT CAGACATGCG ACGGCTTTAG 4150

CCTGGCCTCC TTAAATTCAC CTAAGAATGG GAGCAACCAG CATGCAGGAA 4200

AAGGACAAGC AGCGAAAATT CACGCCCCCT TGGGAGGTGG CGGCATATGC 4250

AAAGGATAGC ACTCCCACTC TACTACTGGG TATCATATGC TGACTGTATA 4300

TGCATGAGGA TAGCATATGC TACCCGGATA CAGATTAGGA TAGCATATAC 4350

TACCCAGATA TAGATTAGGA TAGCATATGC TACCCAGATA TAGATTAGGA 4400

TAGCCTATGC TACCCAGATA TAAATTAGGA TAGCATATAC TACCCAGATA 4450

TAGATTAGGA TAGCATATGC TACCCAGATA TAGATTAGGA TAGCCTATGC 4500

TACCCAGATA TAGATTAGGA TAGCATATGC TACCCAGATA TAGATTAGGA 4550

TAGCATATGC TATCCAGATA TTTGGGTAGT ATATGCTACC CAGATATAAA 4600

TTAGGATAGC ATATACTACC CTAATCTCTA TTAGGATAGC ATATGCTACC 4650

CGGATACAGA TTAGGATAGC ATATACTACC CAGATATAGA TTAGGATAGC 4700

ATATGCTACC CAGATATAGA TTAGGATAGC CTATGCTACC CAGATATAAA 4750

TTAGGATAGC ATATACTACC CAGATATAGA TTAGGATAGC ATATGCTACC 4800

CAGATATAGA TTAGGATAGC CTATGCTACC CAGATATAGA TTAGGATAGC 4850

ATATGCTATC CAGATATTTG GGTAGTATAT GCTACCCATG GCAACATTAG 4900

CCCACCGTGC TCTCAGCGAC CTCGTGAATA TGAGGACCAA CAACCCTGTG 4950

CTTGGCGCTC AGGCGCAAGT GTGTGTAATT TGTCCTCCAG ATCGCAGCAA 5000

58

TCGCGCCCCT ATCTTGGCCC GCCCACCTAC TTATGCAGGT ATTCCCCGGG 5050

GTGCCATTAG TGGTTTTGTG GGCAAGTGGT TTGACCGCAG TGGTTAGCGG 5100

GGTTACAATC AGCCAAGTTA TTACACCCTT ATTTTACAGT CCAAAACCGC 5150

AGGGCGGCGT GTGGGGGCTG ACGCGTGCCC CCACTCCACA ATTTCAAAAA 5200

AAAGAGTGGC CACTTGTCTT TGTTTATGGG CCCCATTGGC GTGGAGCCCC 5250

GTTTAATTTT CGGGGGTGTT AGAGACAACC AGTGGAGTCC GCTGCTGTCG 5300

GCGTCCACTC TCTTTCCCCT TGTTACAAAT AGAGTGTAAC AACATGGTTC 5350

ACCTGTCTTG GTCCCTGCCT GGGACACATC TTAATAACCC CAGTATCATA 5400

TTGCACTAGG ATTATGTGTT GCCCATAGCC ATAAATTCGT GTGAGATGGA 5450

CATCCAGTCT TTACGGCTTG TCCCCACCCC ATGGATTTCT ATTGTTAAAG 5500

ATATTCAGAA TGTTTCATTC CTACACTAGT ATTTATTGCC CAAGGGGTTT 5550

GTGAGGGTTA TATTGGTGTC ATAGCACAAT GCCACCACTG AACCCCCCGT 5600

CCAAATTTTA TTCTGGGGGC GTCACCTGAA ACCTTGTTTT CGAGCACCTC 5650

ACATACACCT TACTGTTCAC AACTCAGCAG TTATTCTATT AGCTAAACGA 5700

AGGAGAATGA AGAAGCAGGC GAAGATTCAG GAGAGTTCAC TGCCCGCTCC 5750

TTGATCTTCA GCCACTGCCC TTGTGACTAA AATGGTTCAC TACCCTCGTG 5800

GAATCCTGAC CCCATGTAAA TAAAACCGTG ACAGCTCATG GGGTGGGAGA 5850

TATCGCTGTT CCTTAGGACC CTTTTACTAA CCCTAATTCG ATAGCATATG 5900

CTTCCCGTTG GGTAACATAT GCTATTGAAT TAGGGTTAGT CTGGATAGTA 5950

TATACTACTA CCCGGGAAGC ATATGCTACC CGTTTAGGGT TAACAAGGGG 6000

GCCTTATAAA CACTATTGCT AATGCCCTCT TGAGGGTCCG CTTATCGGTA 6050

GCTACACAGG CCCCTCTGAT TGACGTTGGT GTAGCCTCCC GTAGTCTTCC 6100

TGGGCCCCTG GGAGGTACAT GTCCCCAGC ATTGGTGTAA GAGCTTCAGC 6150

CAAGAGTTAC ACATAAAGGC AATGTTGTGT TGCAGTCCAC AGACTGCAAA 6200

GTCTGCTCCA GGATGAAAGC CACTCAGTGT TGGCAAATGT GCACATCCAT 6250

TTATAAGGAT GTCAACTACA GTCAGAGAAC CCCTTTGTGT TTGGTCCCCC 6300

CCCGTGTCAC ATGTGGAACA GGGCCCAGTT GGCAAGTTGT ACCAACCAAC 6350

TGAAGGGATT ACATGCACTG CCCGTGACCA ATACAAAACA AAAGCGCTCC 6400

TCGTACCAGC GAAGAAGGGG CAGAGATGCC GTAGTCAGGT TTAGTTCGTC 6450

CGGCGGCGGG GGATCCGCCA GAAATCCGCG CGGTGGTTTT TGGGGGTCGG 6500

GGGTGTTTGG CAGCCACAGA CGCCCGGTGT TCGTGTCGCG CCAGTACATG 6550

CGGTCCATGC CCAGGCCATC CAAAAACCAT GGGTCTGTCT GCTCAGTCCA 6600

GTCGTGGACC TGACCCCACG CAACGCCCAA AAGAATAACC CCCACGAACC 6650

ATAAACCATT CCCCATGGGG GACCCCGTCC CTAACCCACG GGGCCCGTGG 6700

CTATGGCGGG CTTGCCGCCC CGACGTTGGC TGCGAGCCCT GGGCCTTCAC 6750

CCGAACTTGG GGGTTGGGGT GGGGAAAAGG AAGAAACGCG GGCGTATTGG 6800

CCCCAATGGG GTCTCGGTGG GGTATCGACA GAGTGCCAGC CCTGGGACCG 6850

AACCCCGCGT TTATGAACAA ACGACCCAAC ACCCGTGCGT TTTATTCTGT 6900

CTTTTTATTG CCGTCATAGC GCGGGTTCCT TCCGGTATTG TCTCCTTCCG 6950

TGTTTCAGTT AGCCTCCCCC ATCTCCCGGG GTGGGCGAAG AACTCCAGCA 7000

TGAGATCCCC GCGCTGGAGG ATCATCCAGC CGGCGTCCCG GAAAACGATT 7050

CCGAAGCCCA ACCTTTCATA GAAGGCGGCG GTGGAATCGA AATCTCGTGA 7100

TGGCAGGTTG GGCGTCGCTT GGTCGGTCAT TTCGAACCCC AGAGTCCCGC 7150

TCAGAAGAAC TCGTCAAGAA GGCGATAGAA GGCGATGCGC TGCGAATCGG 7200

GAGCGGCGAT ACCGTAAAGC ACGAGGAAGC GGTCAGCCCA TTCGCCGCCA 7250

AGCTCTTCAG CAATATCACG GGTAGCCAAC GCTATGTCCT GATAGCGGTC 7300

CGCCACACCC AGCCGGCCAC AGTCGATGAA TCCAGAAAAG CGGCCATTTT 7350

CCACCATGAT ATTCGGCAAG CAGGCATCGC CATGGGTCAC GACGAGATCC 7400

TCGCCGTCGG GCATGCGCGC CTTGAGCCTG GCGAACAGTT CGGCTGGCGC 7450

GAGCCCCTGA TGCTCTTCGT CCAGATCATC CTGATCGACA AGACCGGCTT 7500

CCATCCGAGT ACGTGCTCGC TCGATGCGAT GTTTCGCTTG GTGGTCGAAT 7550

GGGCAGGTAG CCGGATCAAG CGTATGCAGC CGCCGCATTG CATCAGCCAT 7600

GATGGATACT TTCTCGGCAG GAGCAAGGTG AGATGACAGG AGATCCTGCC 7650

CCGGCACTTC GCGCAATAGC AGCCAGTCCC TTCCCGCTTC AGTGACAACG 7700

TCGAGCACAG CTGCGCAAGG AACGCCCGTC GTGGCCAGCC ACGATAGCCG 7750

CGCTGCCTCG TCCTGCAGTT CATTCAGGGC ACCGGACAGG TCGGTCTTGA 7800

CAAAAAGAAC CGGGCGCCCC TGCGCTGACA GCCGGAACAC GGCGGCATCA 7850

61

GAGCAGCCGA TTGTCTGTTG TGCCCAGTCA TAGCCGAATA GCCTCTCCAC 7900

CCAAGCGGCC GGAGAACCTG CGTGCAATCC ATCTTGTTCA ATCATGCGAA 7950

ACGATCCTCA TCCTGTCTCT TGATCAGATC TGCGGCACGC TGTTGACGCT 8000

GTTAAGCGGG TCGCTGCAGG GTCGCTCGGT GTTCGAGGCC ACACGCGTCA 8050

CCTTAATATG CGAAGTGGAC CTGGGACCGC GCCGCCCCGA CTGCATCTGC 8100

GTGTTCGAAT TCATCAAAGC AACCATAGTA CGCGCCCTGT AGCGGCGCAT 8150

TAAGCGCGGC GGGTGTGGTG GTTACGCGCA GCGTGACCGC TACACTTGCC 8200

AGCGCCCTAG CGCCCGCTCC TTTCGCTTTC TTCCCTTCCT TTCTCGCCAC 8250

GTTCGCCGGC TTTCCCCGTC AAGCTCTAAA TCGGGGGCTC CCTTTAGGGT 8300

TCCGATTTAG TGCTTTACGG CACCTCGACC CCAAAAAACT TGATTTGGGT 8350

GATGGTTCAC GTAGTGGGCC ATCGCCCTGA TAGACGGTTT TTCGCCCTTT 8400

GACGTTGGAG TCCACGTTCT TTAATAGTGG ACTCTTGTTC CAAACTGGAA 8450

CAACACTCAA CCCTATCTCG GGCTATTCTT TTGATTTATA AGGGATTTTG 8500

CCGATTTCGG CCTATTGGTT AAAAAATGAG CTGATTTAAC AAAAATTTAA 8550

CGCGAATTTT AACAAAATAT TAACGTTTAC AATTTTATGG TGCAGGCCTC 8600

GTGATACGCC TATTTTTATA GGTTAATGTC ATGATAATAA TGGTTTCTTA 8650

GACGTCAGGT GGCACTTTTC GGGGAAATGT GCGCGGAACC CCTATTTGTT 8700

TATTTTTCTA AATACATTCA AATATGTATC CGCTCATGAG ACAATAACCC 8750

TGATAAATGC TTCAATAATA TTGAAAAAGG AAGAGTATGA GTATTCAACA 8800

TTTCCGTGTC GCCCTTATTC CCTTTTTTGC GGCATTTTGC CTTCCTGTTT 8850

TTGCTCACCC AGAAACGCTG GTGAAAGTAA AAGATGCTGA AGATCAGTTG 8900

GGTGCACGAG TGGGTTACAT CGAACTGGAT CTCAACAGCG GTAAGATCCT 8950

TGAGAGTTTT CGCCCCGAAG AACGTTTTCC AATGATGAGC ACTTTTAAAG 9000

TTCTGCTATG TGGCGCGGTA TTATCCCGTG ATGACGCCGG GCAAGAGCAA 9050

CTCGGTCGCC GCATACACTA TTCTCAGAAT GACTTGGTTG AGTACTCACC 9100

AGTCACAGAA AAGCATCTTA CGGATGGCAT GACAGTAAGA GAATTATGCA 9150

GTGCTGCCAT AACCATGAGT GATAACACTG CGGCCAACTT ACTTCTGACA 9200

ACGATCGGAG GACCGAAGGA GCTAACCGCT TTTTTGCACA ACATGGGGGA 9250

TCATGTAACT CGCCTTGATC GTTGGGAACC GGAGCTGAAT GAAGCCATAC 9300

CAAACGACGA GCGTGACACC ACGATGCCAG CAGCAATGGC AACAACGTTG 9350

CGCAAACTAT TAACTGGCGA ACTACTTACT CTAGCTTCCC GGCAACAATT 9400

AATAGACTGG ATGGAGGCGG ATAAAGTTGC AGGACCACTT CTGCGCTCGG 9450

CCCTTCCGGC TGGCTGGTTT ATTGCTGATA AATCTGGAGC CGGTGAGCGT 9500

GGGTCTCGCG GTATCATTGC AGCACTGGGG CCAGATGGTA AGCCCTCCCG 9550

TATCGTAGTT ATCTACACGA CGGGGAGTCA GGCAACTATG GATGAACGAA 9600

ATAGACAGAT CGCTGAGATA GGTGCCTCAC TGATTAAGCA TTGGTAACTG 9650

TCAGACCAAG TTTACTCATA TATACTTTAG ATTGATTTAA AACTTCATTT 9700

TTAATTTAAA AGGATCTAGG TGAAGATCCT TTTTGATAAT CTCATGACCA 9750

```
       AAATCCCTTA ACGTGAGTTT TCGTTCCACT GAGCGTCAGA CCCCGTAGAA 9800

       AAGATCAAAG GATCTTCTTG AGATCCTTTT TTTCTGCGCG TAATCTGCTG 9850

       CTTGCAAACA AAAAAACCAC CGCTACCAGC GGTGGTTTGT TTGCCGGATC 9900

       AAGAGCTACC AACTCTTTTT CCGAAGGTAA CTGGCTTCAG CAGAGCGCAG 9950

       ATACCAAATA CTGTCCTTCT AGTGTAGCCG TAGTTAGGCC ACCACTTCAA 10000

       GAACTCTGTA GCACCGCCTA CATACCTCGC TCTGCTAATC CTGTTACCAG 10050

       TGGCTGCTGC CAGTGGCGAT AAGTCGTGTC TTACCGGGTT GGACTCAAGA 10100

       CGATAGTTAC CGGATAAGGC GCAGCGGTCG GGCTGAACGG GGGGTTCGTG 10150

       CACACAGCCC AGCTTGGAGC GAACGACCTA CACCGAACTG AGATACCTAC 10200

       AGCGTGAGCA TTGAGAAAGC GCCACGCTTC CCGAAGGGAG AAAGGCGGAC 10250

       AGGTATCCGG TAAGCGGCAG GGTCGGAACA GGAGAGCGCA CGAGGGAGCT 10300

       TCCAGGGGGA AACGCCTGGT ATCTTTATAG TCCTGTCGGG TTTCGCCACC 10350

       TCTGACTTGA GCGTCGATTT TTGTGATGCT CGTCAGGGGG GCGGAGCCTA 10400

       TGGAAAAACG CCAGCTGGCA CGACAGGTTT CCCGACTGGA AAGCGGGCAG 10450

       TGAGCGCAAC GCAATTAATG TGAGTTACCT CACTCATTAG GCACCCCAGG 10500

       CTTTACACTT TATGCTTCCG GCTCGTATGT TGTGTGGAAT TGTGAGCGGA 10550

       TAACAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG AATTAA 10596


       (2) INFORMATION FOR SEQ ID NO:16:

           (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 51 bases
```

              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

     ATGAAGGCCC CCGCTGTGCT TGCACCTGGC ATCCTCGTGC TCCTGTTTAC 50

10
          C 51

15   (2) INFORMATION FOR SEQ ID NO:17:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 56 bases
              (B) TYPE: nucleic acid
20   (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

25
     CACTAGTTAG GATGGGGGAC ATGTCTGTCA GAGGATACTG CACTTGTCGG 50

     CATGAA 56
30

     (2) INFORMATION FOR SEQ ID NO:18:

35   (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 22 bases
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear
40
          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

     TAGTACTAGC ACTATGATGT CT 22
45

     (2) INFORMATION FOR SEQ ID NO:19:

50   (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 22 bases
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear
55
          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTTACTTCTT GACGGTCCAA AG 22

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 bases
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAGGGGGAGT TGCAGATTCA GCTGT 25

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 bases
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTTTTGTCG GTGACCTGAT CATTCTGATC TGGTTGAACT ATTAC 45

Claims:

1.   A hepatocyte growth factor (HGF) variant resistant to proteolytic cleavage by enzymes that are capable of _in vivo_ conversion of HGF into its two-chain form.

5   2.   The variant of claim 1 which is a variant of human HGF (huHGF):

3.   A hepatocyte growth factor (HGF) variant stabilized in single-chain form by site directed mutagenesis within a region recognized by an enzyme capable of converting HGF into its two-chain
10   form.

4.   The variant of claim 3 which is capable of binding an HGF receptor.

5.   The variant of claim 4 which is a variant of human HGF (huHGF).

15   6.   The variant of claim 5 having an amino acid alteration at or adjacent to amino acid positions 493, 494, 495 or 496 of the wild-type huHGF amino acid sequence.

7.   The variant of claim 6 wherein said alteration is substitution.

20   8.   The variant of claim 6 wherein said alteration is insertion or deletion.

9.   The variant of claim 6 in which amino acid position 494 is occupied by an amino acid other than arginine.

10.   The variant of claim 9 wherein said amino acid is selected
25   from the group consisting of glutamic acid, aspartic acid and alanine.

11.   The variant of claim 6 wherein said alteration is the substitution of at least one amino acid at amino acid positions 493-496 of the wild-type hHGF amino acid sequence.

12.   The variant of claim 11 having another amino acid
30   substituted for arginine at amino acid position 494 of wild-type huHGF.

13.   The variant of claim 11 having another amino acid substituted for valine at amino acid position 495 of wild-type huHGF.

14.   The variant of claim 11 having another amino acid
35   substituted for valine at amino acid position 496 of wild-type huHGF.

15.   The variant of claim 6 retaining substantially full receptor binding affinity of wild-type huHGF.

67

16. A hepatocyte growth factor (HGF) variant having an amino acid alteration at a site within the protease domain of HGF and retaining substantially full receptor binding affinity of the corresponding wild-type HGF.

5   17. The variant of claim 16 comprising an alteration in a region corresponding to the catalytic site of serine proteases.

18. The variant of claim 16 comprising an alteration at or adjacent to any of positions 534, 673 and 692 of the wild-type human HGF (huHGF) amino acid sequence.

10   19. The variant of claim 18 wherein said alteration is substitution.

20. The variant of claim 19 having another amino acid substituted for glutamine at position 534 of the wild-type huHGF amino acid sequence.

15   21. The variant of claim 20 wherein said amino acid is histidine.

22. The variant of claim 19 having another amino acid substituted for tyrosine at position 673 of the wild-type huHGF amino acid sequence.

20   23. The variant of claim 22 wherein said amino acid is devoid of aromatic and heterocyclic moieties.

24. The variant of claim 23 wherein said amino acid is selected from serine, threonine, asparagine, cysteine, glycine, alanine, and valine.

25   25. The variant of claim 24 wherein said amino acid is serine.

26. The variant of claim 19 having another amino acid substituted for valine at position 692 of the wild-type huHGF amino acid sequence.

30   27. The variant of claim 26 wherein said amino acid is polar.

28. The variant of claim 27 wherein said amino acid is selected from serine, threonine, asparagine and glutamine.

29. The variant of claim 28 wherein said amino acid is serine.

30. The variant of claim 24 further comprising the substitution
35 of glutamine at position 534 or valine at position 692 of the wild-type huHGF amino acid sequence.

31. The variant of claim 30 having serine substituted for tyrosine at position 673 of the wild-type huHGF amino acid sequence.

32. The variant of claim 31 having histidine substituted for glutamine at position 534 of the wild-type huHGF amino acid sequence.

33. The variant of claim 31 having serine substituted for valine at position 692 of the wild-type huHGF amino acid sequence.

34. The variant of claim 33 additionally having histidine substituted for glutamine at position 534 of the wild-type huHGF amino acid sequence.

35. The variant of any of claims 1-15 having an amino acid alteration at a site within the protease domain of HGF and retaining substantially full receptor binding affinity of the corresponding wild-type HGF.

36. The variant of any of claims 16-34 that is resistant to proteolytic cleavage.

37. The variant of any of claims 1-36 that is substantially incapable of HGF receptor activation.

38. The variant of any of claims 1-36 that is substantially devoid of HGF hepatocyte growth stimulating activity.

39. The variant of any of claims 1-36 having increased receptor binding affinity as compared to wild-type huHGF.

40. The variant of claim 39 wherein the increase in receptor binding affinity is accomplished by an alteration in a receptor-binding domain of the huHGF amino acid sequence.

41. The variant of claim 40 wherein said alteration is in the huHGF α-chain.

42. The variant of claim 41 wherein said alteration is within the Kringle 1 domain.

43. The variant of claim 42 wherein said alteration is within the patch defined by amino acid positions 159, 161, 195 and 197 of the wild-type huHGF amino acid sequence.

44. The variant of claim 42 wherein said alteration is at amino acid position 173 of wild-type huHGF.

45. The variant of claim 41 wherein said alteration is within the hairpin domain, N-terminal of the hairpin domain, or between the hairpin and the Kringle 1 domains of wild-type huHGF.

46. The variant of any of claims 1-45 devoid of functional Kringle 2 domain.

47. The variant of any of claims 1-45 devoid of functional Kringle 3 domain.

48. The variant of any of claims 1-45 devoid of functional Kringle 4 domain.

49. A nucleotide sequence encoding the variant of any of claims 1-48.

50. A replicable expression vector containing and capable of expressing in a suitable host cell the nucleotide sequence of claim 49.

51. A host cell transformed with the vector of claim 50.

52. A process comprising culturing the host cells of claim 51 so as to express the nucleic acid encoding the HGF variant.

53. The process of claim 52 further comprising recovering the variant from the host cell culture.

54. A pharmaceutical composition comprising a variant of any of claims 1-48 in an amount capable of competitive inhibition of the binding of wild-type huHGF to its receptor, in admixture with a pharmaceutically acceptable carrier.

55. A method of treating a pathological condition associated with the activation of a huHGF receptor comprising administering to a patient in need the composition of claim 54.
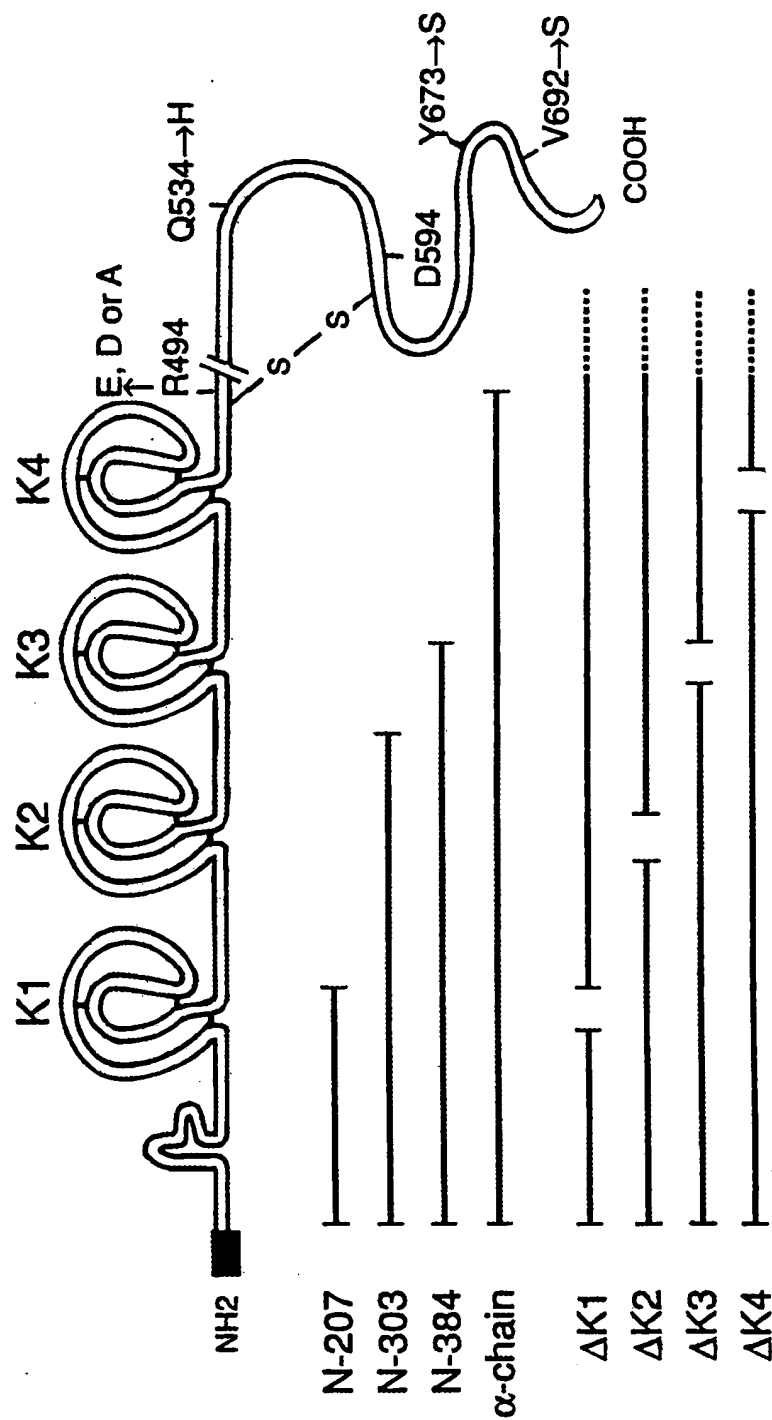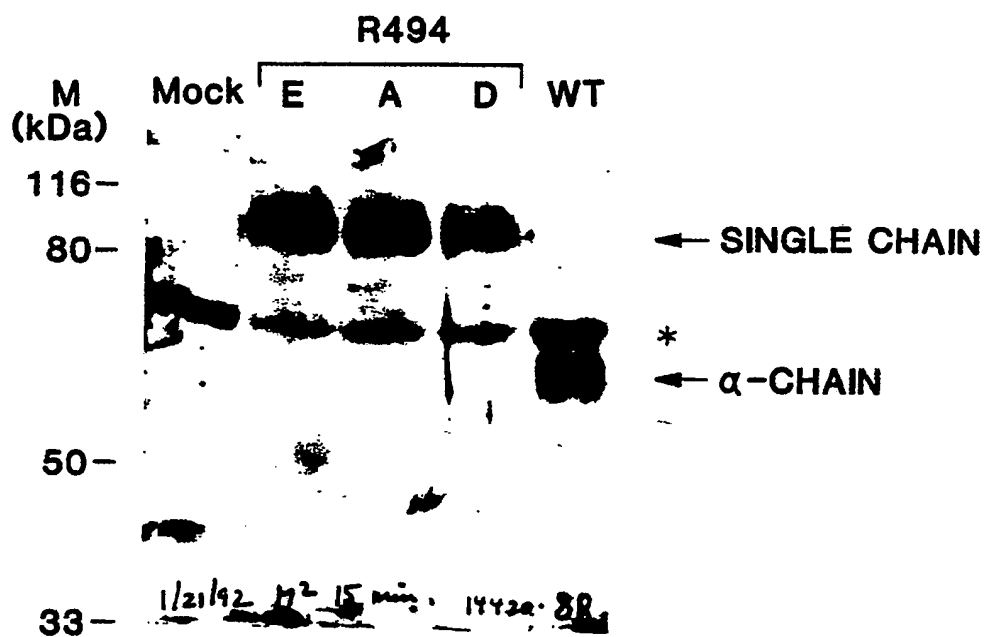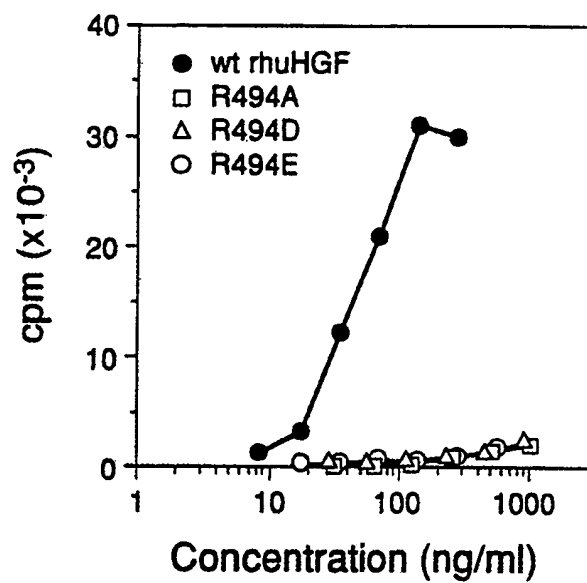
FIG. I

FIG. 2

FIG. 3A

FIG. 3B

FIG. 4

><Modified RK5 derivative, insert Xho I into Hind III site; PJG
2/89>
<from Cori 86.3.18 fix DHFR acc I
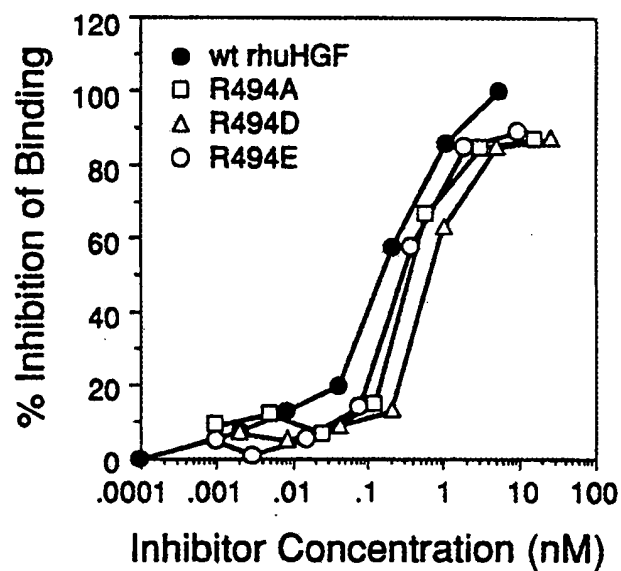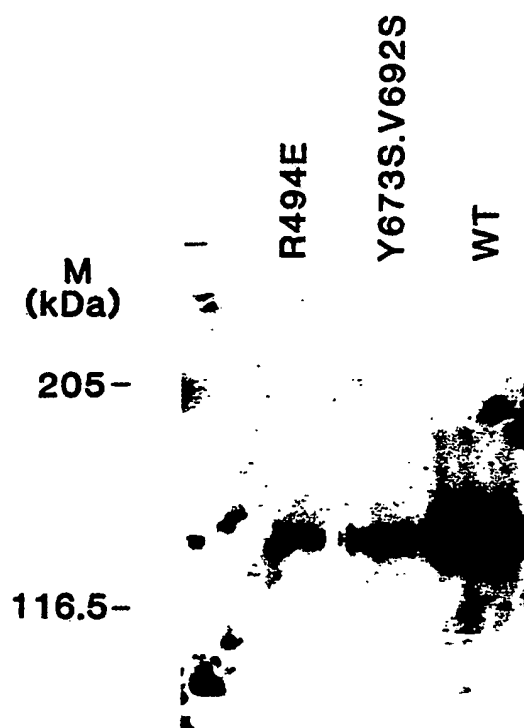<sequence of CMV enhancer/promoter, from Cell 41, 1985>
T><from pPMLCMV beginning to HindIII,enhancers and promoter>
TCGAGCTCGCCCGACATTGATTA
TTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCC
ATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCT
GACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCC
ATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTT
ACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA
CGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCC
CAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATT
AGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGC
GTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGAC
GTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATG
TCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGT
GGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCG
<Begin RNA>
TCAGATCGCCTGG
AGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATC
CAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCA
AGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCT
T
><sp6 promoter>
<GGCCCACCCCCTTGGCTT>CGTTAGAACGCGGCTACAATTAATACATAACC
T T A T G T A T C A T A C A C A T A C G A T T T A G G T G A C A C T A T A > < s p 6   R N A
start>GAATA<ACATCCACTTTGCCTTTC>

ACATCCACTTTGCCTTTCTCTCC
ACAGGTGTCCACTCCCAGGTCCAA<PstI-ClaI converter>CTGCA

>   <   c   l   o   n   i   n   g
linker>CCTCGGTTCTATCGATTGAATTCCCCGGGGATCCTCTAGAGTCGACCTGCAGAAGCTT
GCCTCGAGGCAAGCTT
GGCCGCCATGGCCC
><sv40 early poly A>AACTTGTTTATTGCAGCTTATAATGGTT
ACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTA
GTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCT
GGATCGATCGG
>   <   s   v   4   0
origin>GAATTAATTCGGCGCAGCACCATGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTA
GGTACCTTC
TGAGGCGGAAAGAACCAGCT
GTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGC
AGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGC
TCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCG
CCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCAT
GGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTC
CAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAA
><start pUC118>
AAGCTGTTAACAGCTTGGC
ACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACC
CTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCCTTCGCCAGCTGGCGTAATA

FIG. 5A

GCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGTAGCCTGAATGGCGAATGGC
<start M13>
G CCTGATGCGG
         TATTTTCTCC TTACGCATCT GTGCGGTATT TCACACCGCA TA

CGTCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTG
TGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCG
CTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGG
GGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATT
TGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGT
TGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTA
TCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAA
ATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTT

TATGGTGCAC TCTCAGTACA
         ATCTGCTCTG ATGCCGCATA GTTAAGCCA   ACTCC GCTATCGCTA
CGTGACTGGG
         TCATGGCTGC GCCCCGACAC CCGCCAACAC CCGCTGACGC GCCCTGACGG
GCTTGTCTGC
         TCCCGGCATC CGCTTACAGA CAAGCTGTGA CCGTCTCCGG GAGCTGCATG
TGTCAGAGGT
         TTTCACCGTC ATCACCGAAA CGCGCGAGGC AG
TATTC
<   Hinc II (2271) to GTCATC>
<   Pst I (1973) to CTGCTG>
<   Acc I (183) delete 6 bp>
<Arbitrarily change EcoRI (1) to GAATAC>
<pUCx 83.11.25 sequence not fully known>
                         TTGAAGACGA AAGGGCCTCG TGATACGCCT ATTTTTATAG
         GTTAATGTCA TGATAATAAT GGTTTCTTAG ACGTCAGGTG GCACTTTTCG
GGGAAATGTG
         CGCGGAACCC CTATTTGTTT ATTTTTCTAA ATACATTCAA ATATGTATCC
GCTCATGAGA
         CAATAACCCT GATAAATGCT TCAATAATAT TGAAAAAGGA AGAGTATGAG
TATTCAACAT
         TTCCGTGTCG CCCTTATTCC CTTTTTTGCG GCATTTTGCC TTCCTGTTTT
TGCTCACCCA
         GAAACGCTGG TGAAAGTAAA AGATGCTGAA GATCAGTTGG GTGCACGAGT
GGGTTACATC
         GAACTGGATC TCAACAGCGG TAAGATCCTT GAGAGTTTTC GCCCCGAAGA
ACGTTTTCCA
         ATGATGAGCA CTTTTAAAGT TCTGCTATGT GGCGCGGTAT TATCCCGTGA
TGACGCCGGG
         CAAGAGCAAC TCGGTCGCCG CATACACTAT TCTCAGAATG ACTTGGTTGA
GTACTCACCA
         GTCACAGAAA AGCATCTTAC GGATGGCATG ACAGTAAGAG AATTATGCAG
TGCTGCCATA
         ACCATGAGTG ATAACACTGC GGCCAACTTA CTTCTGACAA CGATCGGAGG
ACCGAAGGAG
         CTAACCGCTT TTTTGCACAA CATGGGGGAT CATGTAACTC GCCTTGATCG
TTGGGAACCG
         GAGCTGAATG AAGCCATACC AAACGACGAG CGTGACACCA CGATGCCAGC
AGCAATGGCA
         ACAACGTTGC GCAAACTATT AACTGGCGAA CTACTTACTC TAGCTTCCCG

FIG. 5B

```
GCAACAATTA
     ATAGACTGGA   TGGAGGCGGA   TAAAGTTGCA   GGACCACTTC   TGCGCTCGGC
CCTTCCGGCT
     GGCTGGTTTA   TTGCTGATAA   ATCTGGAGCC   GGTGAGCGTG   GGTCTCGCGG
TATCATTGCA
     GCACTGGGGC   CAGATGGTAA   GCCCTCCCGT   ATCGTAGTTA   TCTACACGAC
GGGGAGTCAG
     GCAACTATGG   ATGAACGAAA   TAGACAGATC   GCTGAGATAG   GTGCCTCACT
GATTAAGCAT
     TGGTAACTGT   CAGACCAAGT   TTACTCATAT   ATACTTTAGA   TTGATTTAAA
ACTTCATTTT
     TAATTTAAAA   GGATCTAGGT   GAAGATCCTT   TTTGATAATC   TCATGACCAA
AATCCCTTAA
     CGTGAGTTTT   CGTTCCACTG   AGCGTCAGAC   CCCGTAGAAA   AGATCAAAGG
ATCTTCTTGA
     GATCCTTTTT   TTCTGCGCGT   AATCTGCTGC   TTGCAAACAA   AAAAACCACC
GCTACCAGCG
     GTGGTTTGTT   TGCCGGATCA   AGAGCTACCA   ACTCTTTTTC   CGAAGGTAAC
TGGCTTCAGC
     AGAGCGCAGA   TACCAAATAC   TGTCCTTCTA   GTGTAGCCGT   AGTTAGGCCA
CCACTTCAAG
     AACTCTGTAG   CACCGCCTAC   ATACCTCGCT   CTGCTAATCC   TGTTACCAGT
GGCTGCTGCC
     AGTGGCGATA   AGTCGTGTCT   TACCGGGTTG   GACTCAAGAC   GATAGTTACC
GGATAAGGCG
     CAGCGGTCGG   GCTGAACGGG   GGGTTCGTGC   ACACAGCCCA   GCTTGGAGCG
AACGACCTAC
     ACCGAACTGA   GATACCTACA   GCGTGAGCAT   TGAGAAAGCG   CCACGCTTCC
CGAAGGGAGA
     AAGGCGGACA   GGTATCCGGT   AAGCGGCAGG   GTCGGAACAG   GAGAGCGCAC
GAGGGAGCTT
     CCAGGGGGAA   ACGCCTGGTA   TCTTTATAGT   CCTGTCGGGT   TTCGCCACCT
CTGACTTGAG
     CGTCGATTTT   TGTGATGCTC   GTCAGGGGGG   CGGAGCCTAT   GGAAAAACGC
CAGCAACGCG
     GCCTTTTTAC   GGTTCCTGGC   CTTTTGCTGG   CCTTTTGCTC   ACATGTTCTT
TCCTGCGTTA
     TCCCCTGATT   CTGTGGATAA   CCGTATTACC   GCCTTTGAGT   GAGCTGATAC
CGCTCGCCGC
     AGCCGAACGA   CCGAGCGCAG   CGAGTCAGTG   AGCGAGGAAG   CGGAAGAGC
<end M13>
GCCCAATACGCAA
ACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATCCAGCTGGCACGACAGGTTTCCCGA
CTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTACCTCACTCATTAGGCACC
CCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA
ATTTCACACAGGAAACAGCTATGACCATGATTAC
GAATTAA
```

<p style="text-align:center;">FIG. 5C</p>

```
><pCIS.EBON
>< assembled by Steve Williams June 1989
><"poison-minus" pRK
><with EBNA-1, oriP, neoR
><polylinker sites:  XhoI, HindIII, NotI

><CMV enhancer/promoter
T
TCGAGCTCGCCCGACATTGATTA
TTG<SpeI>ACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCC
ATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCT
GACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCC
ATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTT
ACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA
CGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCC
CAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATT
AGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGC
GTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGAC
GTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATG
TCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGT
GGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCG
<Begin RNA>
TCAGATCGCCTGG
AGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATC
CAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCA
AGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCT
T
><sp6 promoter>
<GGCCCACCCCCTTGGCTT>CGTTAGAACGCGGCTACAATTAATACATAACC
TTATGTATCATACACATACGATTTAGGTGACACTATA><sp6      RNA
start>GAATA<ACATCCACTTTGCCTTTC>

ACATCCACTTTGCCTTTCTCTCC
ACAGGTGTCCACTCCCAGGTCCAA<PstI-ClaI converter>CTGCA

><cloning linker>CCTCGGTTCT
ATCGATTCTCGA

<EcoRI/klenow>GAATTAATTC

AAGCTTGCGGCCGCAGCTT
GGCCGCCATGGCCC
><sv40 early poly A>AACTTGTTTATTGCAGCTTATAATGGTT
ACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTA
GTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCT
GGATCGATCGG
>        <        s        v        4        0
origin>GAATTAATTCGGCGCAGCACCATGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTA

<KpnI>GGTACCTTC
TGAGGCGGAAAGAACCAGCT
GTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGC
AGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGC
```

FIG. 6A

TCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCG
CCCCTAACTCCGCCCATCCCGCCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCAT
GGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTC
CAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAA
 <start pUC118>
AAGCTGTT<HpaI end from SW1>

><delta 3
 <PCR primer seq; PCR product was blunted & ligated in> CACGTGAT
<this is EcoRV site remnant>

<RI cassette: EBNA-oriP-tk term-NeoR-tk prom>
<4913 bp EcoRI-Bam from 220_2>
<EcoRI>
 GAATTCTCAT
 GTTTGACAGC TTATCATCGA TA

><EBNA-1>
 GATCCTCACA GGCCGCACCC AGCTTTTCTT CCGTTGCCCC AGTAGCATCT CTGTCTGGTG
 ACCTTGAAGA GGAAGAGGAG GGGTCCCGAG AATCCCCATC CCTACCGTCC AGCAAAAAGG
 GGGACGAGGA ATTTGAGGCC TGGCTTGAGG CTCAGGACGC AAATCTTGAG GATGTTCAGC
 GGGAGTTTTC CGGGCTGCGA GTAATTGGTG ATGAGGACGA GGATGGTTCG GAGGATGGGG
 AATTTTCAGA CCTGGATCTG TCTGACAGCG ACCATGAAGG GGATGAGGGT GGGGGGGCTG
 TTGGAGGGGG CAGGAGTCTG CACTCCCTGT ATTCACTGAG CGTCGTCTAA TAAAGATGTC
 TATTGATCTC TTTTAGTGTG AATCATGTCT GACGAGGGGC CAGGTACAGG ACCTGGAAAT
 GGCCTAGGAG AGAAGGGAGA CACATCTGGA CCAGAAGGCT CCGGCGGCAG TGGACCTCAA
 AGAAGAGGGG GTGATAACCA TGGACGAGGA CGGGGAAGAG GACGAGGACG AGGAGGCGGA
 AGACCAGGAG CCCCGGGCGG CTCAGGATCA GGGCCAAGAC ATAGAGATGG TGTCCGGAGA
 CCCCAAAAAC GTCCAAGTTG CATTGGCTGC AAAGGGACCC ACGGTGGAAC AGGAGCAGGA
 GCAGGAGCGG GAGGGGCAGG AGCAGGAGGG GCAGGAGCAG GAGGAGGGGC AGGAGCAGGA
 GGAGGGGCAG GAGGGGCAGG AGGGGCAGGA GGGGCAGGAG CAGGAGGAGG GGCAGGAGCA
 GGAGGAGGGG CAGGAGGGGC AGGAGGGGCA GGAGCAGGAG GAGGGGCAGG AGCAGGAGGA
 GGGGCAGGAG GGGCAGGAGC AGGAGGAGGG GCAGGAGGGG CAGGAGGGGC AGGAGCAGGA
 GGAGGGGCAG GAGCAGGAGG AGGAGGGGCA GGAGCAGGAG GGGCAGGAGG GGCAGGAGGG
 GCAGGAGGGG CAGGAGCAGG AGGAGGGGCA GGAGCAGGAG GGGCAGGAGG GGCAGGAGCA
 GGAGGGGCAG GAGCAGGAGG GGCAGGAGCA GGAGGGGCAG GAGCAGGAGG GGCAGGAGGG
 GCAGGAGCAG GAGGGGCAGG AGGGGCAGGA GCAGGAGGGG CAGGAGGGGC AGGAGCAGGA
 GGAGGGGCAG GAGGGGCAGG AGCAGGAGGA GGGGCAGGAG GGGCAGGAGC AGGAGGGGCA
 GGAGGGGCAG GAGCAGGAGG GGCAGGAGGG GCAGGAGCAG GAGGGGCAGG AGGGGCAGGA
 GCAGGAGGAG GGGCAGGAGC AGGAGGGGCA GGAGCAGGAG GTGGAGGCCG GGGTCGAGGA
 GGCAGTGGAG GCCGGGGTCG AGGAGGTAGT GGAGGCCGGG GTCGAGGAGG TAGTGGAGGC
 CGCCGGGGTA GAGGACGTGA AAGAGCCAGG GGGGGAAGTC GTGAAAGAGC CAGGGGGAGA
 GGTCGTGGAC GTGGAGAAAA GAGGCCCAGG AGTCCCAGTA GTCAGTCATC ATCATCCGGG
 TCTCCACCGC GCAGGCCCCC TCCAGGTAGA AGGCCATTTT TCCACCCTGT AGGGGAAGCC
 GATTATTTTG AATACCACCA AGAAGGTGGC CCAGATGGTG AGCCTGACGT GCCCCCGGGA
 GCGATAGAGC AGGGCCCCGC AGATGACCCA GGAGAAGGCC CAAGCACTGG ACCCCGGGGT
 CAGGGTGATG GAGGCAGGCG CAAAAAAGGA GGGTGGTTTG GAAAGCATCG TGGTCAAGGA
 GGTTCCAACC CGAAATTTGA GAACATTGCA GAAGGTTTAA GAGCTCTCCT GGCTAGGAGT
 CACGTAGAAA GGACTACCGA CGAAGGAACT TGGGTCGCCG GTGTGTTCGT ATATGGAGGT
 AGTAAGACCT CCCTTTACAA CCTAAGGCGA GGAACTGCCC TTGCTATTCC ACAATGTCGT
 CTTACACCAT TGAGTCGTCT CCCCTTTGGA ATGGCCCCTG GACCCGGCCC ACAACCTGGC
 CCGCTAAGGG AGTCCATTGT CTGTTATTTC ATGGTCTTTT TACAAACTCA TATATTTGCT
 GAGGTTTTGA AGGATGCGAT TAAGGACCTT GTTATGACAA AGCCCGCTCC TACCTGCAAT

FIG. 6B

```
ATCAGGGTGA CTGTGTGCAG CTTTGACGAT GGAGTAGATT TGCCTCCCTG GTTTCCACCT
ATGGTGGAAG GGGCTGCCGC GGAGGGTGAT GACGGAGATG ACGGAGATGA AGGAGGTGAT
GGAGATGAGG GTGAGGAAGG GCAGGAGTGA TGTAACTTGT TAGGAGACGC CCTCAATCGT
ATTAAAAGCC GTGTATTCCC CCGCACTAAA GAATAAATCC CCAGTAGACA TCATGCGTGC
TGTTGGTGTA TTTCTGGCCA TCTGTCTTGT CACCATTTTC GTCCTCCCAA CATGGGGCAA
TTGGGCATAC CCATGTTGTC ACGTCACTCA GCTCCGCGCT CAACACCTTC TCGCGTTGGA
AAACATTAGC GACATTTACC TGGTGAGCAA TCAGACATGC GACGGCTTTA GCCTGGCCTC
CTTAAATTCA CCTAAGAATG GGAGCAACCA
                    ><oriP>GCATGCAGGA AAAGGACAAG CAGCGAAAAT
TCACGCCCCC TTGGGAGGTG GCGGCATATG CAAAGGATAG CACTCCCACT CTACTACTGG
GTATCATATG CTGAC
><family of repeats>
                    TGTAT ATGCATGAGG ATAGCATATG CTACCCGGAT ACAGATTAGG
ATAGCATATA CTACCCAGAT ATAGATTAGG ATAGCATATG CTACCCAGAT ATAGATTAGG
ATAGCCTATG CTACCCAGAT ATAAATTAGG ATAGCATATA CTACCCAGAT ATAGATTAGG
ATAGCATATG CTACCCAGAT ATAGATTAGG ATAGCCTATG CTACCCAGAT ATAGATTAGG
ATAGCATATG CTACCCAGAT ATAGATTAGG ATAGCATATG CTATCCAGAT ATTTGGGTAG
TATATGCTAC CCAGATATAA ATTAGGATAG CATATACTAC CCTAATCTCT ATTAGGATAG
CATATGCTAC CCGGATACAG ATTAGGATAG CATATACTAC CCAGATATAG ATTAGGATAG
CATATGCTAC CCAGATATAG ATTAGGATAG CCTATGCTAC CCAGATATAA ATTAGGATAG
CATATACTAC CCAGATATAG ATTAGGATAG CATATGCTAC CCAGATATAG ATTAGGATAG
CCTATGCTAC CCAGATATAG ATTAGGATAG CATATGCTAT CCAGATATTT GGGTAGTATA
TGCTACCC
><end family of repeats>
                    AT GGCAACATTA GCCCACCGTG CTCTCAGCGA CCTCGTGAAT ATGAGGACCA
ACAACCCTGT GCTTGGCGCT CAGGCGCAAG TGTGTGTAAT TTGTCCTCCA GATCGCAGCA
ATCGCGCCCC TATCTTGGCC CGCCCACCTA CTTATGCAGG TATTCCCCGG GGTGCCATTA
GTGGTTTTGT GGGCAAGTGG TTTGACCGCA GTGGTTAGCG GGGTTACAAT CAGCCAAGTT
ATTACACCCT TATTTTACAG TCCAAAACCG CAGGGCGGCG TGTGGGGGCT GACGCGTGCC
CCCACTCCAC AATTTCAAAA AAAAGAGTGG CCACTTGTCT TTGTTTATGG GCCCCATTGG
CGTGGAGCCC CGTTTAATTT TCGGGGGTGT TAGAGACAAC CAGTGGAGTC CGCTGCTGTC
GGCGTCCACT CTCTTTCCCC TTGTTACAAA TAGAGTGTAA CAACATGGTT CACCTGTCTT
GGTCCCTGCC TGGGACACAT CTTAATAACC CCAGTATCAT ATTGCACTAG GATTATGTGT
TGCCCATAGC CATAAATTCG TGTGAGATGG ACATCCAGTC TTTACGGCTT GTCCCCACCC
CATGGATTTC TATTGTTAAA GATATTCAGA ATGTTTCATT CCTACACTAG TATTTATTGC
CCAAGGGGTT TGTGAGGGTT ATATTGGTGT CATAGCACAA TGCCACCACT GAACCCCCCG
TCCAAATTTT ATTCTGGGGG CGTCACCTGA AACCTTGTTT TCGAGCACCT CACATACACC
TTACTGTTCA CAACTCAGCA GTTATTCTAT TAGCTAAACG AAGGAGAATG AAGAAGCAGG
CGAAGATTCA GGAGAGTTCA CTGCCCGCTC CTTGATCTTC AGCCACTGCC CTTGTGACTA
AAATGGTTCA CTACCCTCGT GGAATCCTGA CCCCATGTAA ATAAAACCGT GACAGCTCAT
GGGGTGGGAG ATATCGCTGT TCCTTA
                    ><dyad region>GGAC CCTTTTACTA ACCCTAATTC GATAGCATAT
GCTTCCCGTT GGGTAACATA TGCTATTGAA TTAGGGTTAG TCTGGATAGT ATATACTACT
ACCCGGGAAG CATATGCTA
                    ><end dyad>C CCGTTTAGGG TTAACAAGGG GGCCTTATAA ACACTATTGC
TAATGCCCTC TTGAGGGTCC GCTTATCGGT AGCTACACAG GCCCCTCTGA TTGACGTTGG
TGTAGCCTCC CGTAGTCTTC CTGGGCCCCT GGGAGGTACA TGTCCCCCAG CATTGGTGTA
AGAGCTTCAG CCAAGAGTTA CACATAAAGG CAATGTTGTG TTGCAGTCCA CAGACTGCAA
AGTCTGCTCC AGGATGAAAG CCACTCAGTG TTGGCAAATG TGCACATCCA TTTATAAGGA
TGTCAACTAC AGTCAGAGAA CCCCTTTGTG TTTGGTCCCC CCCCGTGTCA CATGTGGAAC
AGGGCCCAGT TGGCAAGTTG TACCAACCAA CTGAAGGGAT TACATGCACT GCCCG
><end oriP>
                                ><HSV TK TERM 3'END>TGACC
AATACAAAAC AAAAGCGCTC CTCGTACCAG CGAAGAAGGG GCAGAGATGC CGTAGTCAGG
```

FIG. 6C

```
                TTTAGTTCGT CCGGCGGC
                ><pUC12 SmaI-HaeIII polylinker>
                                  GG G<Bam site is next>


                <1.65 kb Bam-EcoRI frag from pkan2.pcr.bam>
                GGATCC
                ><Bam site made by PCR>
                GCCAGAAATCCGCGCGGTGGTTTTTGGGGGTCGG
                GGGTGTTTGGCAGCCACAGACGCCCGGTGTTCGTGTCGCGCCAGTACATGCGGTCCATGC
                CCAGGCCATCCAAAAACCATGGGTCTGTCTGCTCAGTCCAGTCGTGGACCTGACCCCACG
                CAACGCCCAAAAGAATAACCCCCACGAACCATAAACCATTCCCCATGGGGGACCCCGTCC
                CTAACCCACGGGGCCCGTGGCTATGGCGGGCTTGCCGCCCCGACGTTGGCTGCGAGCCCT
                GGGCCTTCACCCGAACTTGGGGGTTGGGGTGGGGAAAAGGAAGAAACGCGGGCGTATTGG
                CCCCAATGGGGTCTCGGTGGGGTATCGACAGAGTGCCAGCCCTGGGACCGAACCCCGCGT
                TTATGAACAAACGACCCAACACCCGTGCGTTTTATTCTGTCTTTTTATTGCCGTCATAGC
                GCGGGTTCCTTCCGGTATTGTCTCCTTCCGTGTTTCAGTTAGCCTCCCCCATCT
                <HSV1 tk terminater SmaI>

                 <following is EcoRI - SmaI from pKan2, rc>
                ><tn5 neomycin phosphotransferase gene>
                 <BglII-Sma, rc>
                <SmaI,AvaI>CCCGGGGTGGGCGAAGAACTCCAGCATGAGATCCCCGCGCT
                GGAGGATCATCCAGCCGGCGTCCCGGAAAACGATTCCGAAGCCCAACCTTTCATAGAAGG
                CGGCGGTGGAATCGAAATCTCGTGATGGCAGGTTGGGCGTCGCTTGGTCGGTCATTTCGA
                ACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGA
                ATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTC
                TTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCG
                GCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGC
                ATCGCCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAA
                CAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACC
                GGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCA
                GGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTC
                GGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCA
                GTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGC
                CAGCCACGATAGCCGCGCTGCCTCGTCCTGCAGTTCATTCAGGGCACCGGACAGGTCGGT
                CTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCA
                GCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGA
                ACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATCCTCATCCTGTCTCTTGATC

                ><tk promoter>
                 <EcoRI-BglII, rc>
                <BglII>AGATCTGCGGCACGCTGTTGACGCTGTTAAGCGGGTCGCTGCA
                GGGTCGCTCGGTGTTCGAGGCCACACGCGTCACCTTAATATGCGAAGTGGACCTGGGACC
                GCGCCGCCCCGACTGCATCTGCGTGTTCGAATTC<EcoRI>


                <back to SW2 sequence; EcoRV site remnant>
                A

                ><M13 ori>
                        TCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTG
                TGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCG
```

FIG. 6D

CTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGG
GGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATT
TGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGT
TGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTA
TCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAA
ATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTT

TATGGTGCA<ApaLI/blunt>

><delta 2a>

		<EcoO109I/blunt>GGCCTCG		TGATACGCCT
ATTTTTATAG				
GTTAATGTCA	TGATAATAAT	GGTTCTTAG	ACGTCAGGTG	GCACTTTTCG
GGGAAATGTG				
CGCGGAACCC	CTATTTGTTT	ATTTTTCTAA	ATACATTCAA	ATATGTATCC
GCTCATGAGA				
CAATAACCCT	GATAAATGCT	TCAATAATAT	TGAAAAAGGA	AGAGTATGAG
TATTCAACAT				
TTCCGTGTCG	CCCTTATTCC	CTTTTTTGCG	GCATTTTGCC	TTCCTGTTTT
TGCTCACCCA				
GAAACGCTGG	TGAAAGTAAA	AGATGCTGAA	GATCAGTTGG	GTGCACGAGT
GGGTTACATC				
GAACTGGATC	TCAACAGCGG	TAAGATCCTT	GAGAGTTTTC	GCCCCGAAGA
ACGTTTTCCA				
ATGATGAGCA	CTTTTAAAGT	TCTGCTATGT ·	GGCGCGGTAT	TATCCCGTGA
TGACGCCGGG				
CAAGAGCAAC	TCGGTCGCCG	CATACACTAT	TCTCAGAATG	ACTTGGTTGA
GTACTCACCA				
GTCACAGAAA	AGCATCTTAC	GGATGGCATG	ACAGTAAGAG	AATTATGCAG
TGCTGCCATA				
ACCATGAGTG	ATAACACTGC	GGCCAACTTA	CTTCTGACAA	CGATCGGAGG
ACCGAAGGAG				
CTAACCGCTT	TTTTGCACAA	CATGGGGGAT	CATGTAACTC	GCCTTGATCG
TTGGGAACCG				
GAGCTGAATG	AAGCCATACC	AAACGACGAG	CGTGACACCA	CGATGCCAGC
AGCAATGGCA				
ACAACGTTGC	GCAAACTATT	AACTGGCGAA	CTACTTACTC	TAGCTTCCCG
GCAACAATTA				
ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	GGACCACTTC	TGCGCTCGGC
CCTTCCGGCT				
GGCTGGTTTA	TTGCTGATAA	ATCTGGAGCC	GGTGAGCGTG	GGTCTCGCGG
TATCATTGCA				
GCACTGGGGC	CAGATGGTAA	GCCCTCCCGT	ATCGTAGTTA	TCTACACGAC
GGGGAGTCAG				
GCAACTATGG	ATGAACGAAA	TAGACAGATC	GCTGAGATAG	GTGCCTCACT
GATTAAGCAT				
TGGTAACTGT	CAGACCAAGT	TTACTCATAT	ATACTTTAGA	TTGATTTAAA
ACTTCATTTT				
TAATTTAAAA	GGATCTAGGT	GAAGATCCTT	TTTGATAATC	TCATGACCAA
AATCCCTTAA				
CGTGAGTTTT	CGTTCCACTG	AGCGTCAGAC	CCCGTAGAAA	AGATCAAAGG
ATCTTCTTGA				
GATCCTTTTT	TTCTGCGCGT	AATCTGCTGC	TTGCAAACAA	AAAAACCACC

FIG. 6E

```
GCTACCAGCG
     GTGGTTTGTT   TGCCGGATCA   AGAGCTACCA   ACTCTTTTTC   CGAAGGTAAC
TGGCTTCAGC
     AGAGCGCAGA   TACCAAATAC   TGTCCTTCTA   GTGTAGCCGT   AGTTAGGCCA
CCACTTCAAG
     AACTCTGTAG   CACCGCCTAC   ATACCTCGCT   CTGCTAATCC   TGTTACCAGT
GGCTGCTGCC
     AGTGGCGATA   AGTCGTGTCT   TACCGGGTTG   GACTCAAGAC   GATAGTTACC
GGATAAGGCG
     CAGCGGTCGG   GCTGAACGGG   GGGTTCGTGC   ACACAGCCCA   GCTTGGAGCG
AACGACCTAC
     ACCGAACTGA   GATACCTACA   GCGTGAGCAT   TGAGAAAGCG   CCACGCTTCC
CGAAGGGAGA
     AAGGCGGACA   GGTATCCGGT   AAGCGGCAGG   GTCGGAACAG   GAGAGCGCAC
GAGGGAGCTT
     CCAGGGGGAA   ACGCCTGGTA   TCTTTATAGT   CCTGTCGGGT   TTCGCCACCT
CTGACTTGAG
     CGTCGATTTT   TGTGATGCTC   GTCAGGGGGG   CGGAGCCTAT   GGAAAAACGC  CAG
```

><delta1.PVU>
 <PvuII site introduced by mutagenesis; 228 bp PvuII fragment
 deleted>
 <join to PvuII at 4532 in RK5>
```
                                         CTGGCACGACAGGTTTCCCGA
CTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTACCTCACTCATTAGGCACC
CCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA
ATTTCACACAGGAAACAGCTATGACCATGATTAC
GAATTAA
```

FIG. 6F

I. CLASSIFICATION OF SUBJECT MATTER   (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl. 5 C12N15/19;   C07K13/00;   A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]		
Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 205 184 (THE UNITED STATES OF AMERICA) 2 April 1992 see page 4, line 1 - page 6, line 6 see page 10, line 22 - page 11, line 14 see page 13, line 7 - page 14, line 11 ---	1,2, 37-41, 46-55
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 172, no. 1, 15 October 1990, DULUTH, MINNESOTA US pages 321 - 327 TATSUYA, SEKI ET AL. 'Isolation and expression of cDNA for different forms of hepatocyte growth factor from human leukocyte' cited in the application see page 322, paragraph 1; figure 2 see page 326, paragraph 1 --- -/--	16,35, 41-43, 49-53

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 AUGUST 1993	23. 09. 93
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 180, no. 2, 31 October 1991, DULUTH, MINNESOTA US pages 1151 - 1158 NOBUYUKI SHIMA ET AL. 'Tumor cytotoxic factor / hepatocyte growth factor from human fibroblasts: cloning of its cDNA, purification and characterization of recombinant protein' see abstract see page 1151, paragraph 1 - page 1152, paragraph 1; figure 1 see page 1154, paragraph 2 - page 1157, paragraph 1 ---	16,35, 41-43, 49-53
P,X	EMBO JOURNAL vol. 11, no. 7, July 1992, EYNSHAM, OXFORD GB pages 2503 - 2510 NATHALIE A. LOKKER ET AL. 'Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding' see abstract see page 2505, left column, paragraph 3 - page 2508, right column, paragraph 1 ---	1-7, 9-12, 15-41, 46-53
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 28, 5 October 1992, BALTIMORE, MD US pages 20114 - 20119 DAIJI NAKA ET AL. 'Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer' see abstract see page 20114, left column, paragraph 2 - right column, paragraph 2 see page 20116, right column, paragraph 2 - page 20118, right column, paragraph 2; figure 4 ---	1-7,9, 11,12, 15-17, 36,37, 49-53

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	FEBS LETTERS. vol. 311, no. 1, October 1992, AMSTERDAM NL pages 17 - 21 EVA GAK ET AL. 'Processing of hep: tocyte growth factor to the heterodimeric form is required for biological activity' see page 17, left column, paragraph 2 - right column, paragraph 1 see page 18, right column, paragraph 2 - page 20, left column, paragraph 1 -----	1-7, 9-12,15, 36-39, 49-53

I. International application No.

PCT/US 93/04648

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 55 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 27/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9205184	02-04-92	AU-A- 8659591	15-04-92
		CA-A- 2091700	15-03-92